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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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[X] *Additional inventors are being named on the* 2 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

M-CSF-SPECIFIC MONOCLONAL ANTIBODY AND USES THEREOF

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ENCLOSED APPLICATION PARTS (*check all that apply*)

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[ ] Application Data Sheet. See 37 CFR 1.76			

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Respectfully submitted,

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV 323768015US, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450, on the date shown below.

Dated: June 2, 2004 Signature: _____ (Richard Zimmermann)

PROVISIONAL APPLICATION COVER SHEET
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
PROVISIONAL APPLICATION FOR UNITED STATES LETTERS PATENT

Title:

M-CSF-SPECIFIC MONOCLONAL ANTIBODY AND USES THEREOF

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M-CSF-SPECIFIC MONOCLONAL ANTIBODY AND USES THEREOF

Related subject matter is disclosed in U.S. Provisional Application No. 60/535,181, filed January 7, 2004 [Attorney Docket No. PP21601.001-27527/39771], which is incorporated by reference herein in its entirety.

TECHNICAL FIELD

This invention relates to methods for preventing and treating cancer metastasis and bone loss associated with cancer metastasis by administering an M-CSF-specific antibody to a subject.

BACKGROUND OF THE INVENTION

Cancer metastasis is the primary cause of post-operation or post-therapy recurrence in cancer patients. Despite intensive efforts to develop treatments, cancer metastasis remains substantially refractory to therapy. Bone is one of the most common sites of metastasis of various types of human cancers (e.g., breast, lung, prostate and thyroid cancers). The occurrence of osteolytic bone metastases causes serious morbidity due to intractable pain, high susceptibility to fracture, nerve compression and hypercalcemia. Despite the importance of these clinical problems, there are few available treatments for bone loss associated with cancer metastasis.

Osteoclasts mediate bone readsorption. Osteoclasts are multinucleated cells differentiating from haemopoietic cells. It is generally accepted that osteoclasts are formed by the fusion of mononuclear precursors derived from haemopoietic stem cells in the bone marrow, rather than incomplete cell divisions (Chambers, Bone and Mineral Research, 6: 1-25, 1989; Göthling et al., Clin Orthop Relat R. 120: 201-228, 1976; Kahn et al., Nature 258: 325-327, 1975, Suda et al., Endocr Rev 13: 66-80, 1992; Walker, Science 180: 875, 1973; Walker, Science 190: 785-787, 1975; Walker, Science 190: 784-785, 1975). They share a common stem cell with monocyte-macrophage lineage cells (Ash et al., Nature 283: 669-670, 1980, Kerby et al., J. Bone Miner Res 7: 353-62, 1992). The differentiation of osteoclast precursors into mature multinucleated osteoclasts requires different factors including hormonal and local stimuli (Athanasou et al., Bone Miner 3: 317-333, 1988; Feldman et al., Endocrinology 107: 1137-1143, 1980; Walker, Science 190: 784-785, 1975; Zheng et al., Histochem J 23: 180-188, 1991) and living bone and bone cells have been shown to play a critical role in osteoclast development (Hagenaars et al., Bone Miner 6: 179-189, 1989). Osteoblastic or bone marrow stromal cells are also required for osteoclast differentiation.

One of the factors produced by these cells that supports osteoclast formation is macrophage-colony stimulating factor, M-CSF (Wiktor-Jedrzejczak et al., Proc Natl Acad Sci USA 87: 4828-4832, 1990; Yoshida et al., Nature 345: 442-444, 1990). Receptor activator for NF-κ B ligand (RANKL, also known as TRANCE, ODF and OPGL) is another signal (Suda et al., Endocr Rev 13: 66-80, 1992) through which osteoblastic/stromal cells stimulate osteoclast formation and resorption via a receptor, RANK (TRANCER), located on osteoclasts and osteoclast precursors (Lacey et al., Cell 93: 165-176, 1998; Tsuda et al., Biochem Biophys Res Co 234: 137-142, 1997; Wong et al., J Exp Med 186: 2075-2080, 1997; Wong et al., J Biol. Chem 272: 25190-25194, 1997; Yasuda et al., Endocrinology 139: 1329-1337, 1998; Yasuda et al., Proc Natl Acad Sci US 95: 3597-3602, 1998). Osteoblasts also secrete a protein that strongly inhibits osteoclast formation called osteoprotegerin (OPG, also known as OCIF), which acts as a decoy receptor for the RANKL thus inhibiting the positive signal between osteoclasts and osteoblasts via RANK and RANKL.

Osteoclasts are responsible for dissolving both the mineral and organic bone matrix (Blair et al., J Cell Biol 102: 1164-1172, 1986). Osteoclasts represent terminally differentiated cells expressing a unique polarized morphology with specialized membrane areas and several membrane and cytoplasmic markers, such as tartrate resistant acid phosphatase (TRAP) (Anderson et al. 1979), carbonic anhydrase II (Väänänen et al., Histochemistry 78: 481-485, 1983), calcitonin receptor (Warshafsky et al., Bone 6: 179-185, 1985) and vitronectin receptor (Davies et al., J Cell Biol 109: 1817-1826, 1989). Multinucleated osteoclasts usually contain less than 10 nuclei, but they may contain up to 100 nuclei being between 10 and 100 μm in diameter (Göthling et al., Clin Orthop Relat R 120: 201-228, 1976). This makes them relatively easy to identify by light microscopy. They are highly vacuolated when in the active state, and also contain many mitochondria, indicative of a high metabolic rate (Mundy, in Primer on the metabolic bone diseases and disorders of mineral metabolism, pages 18-22, 1990). Since osteoclasts play a major role in osteolytic bone metastases, there is a need in the art for new agents and methods for preventing osteoclast stimulation and function.

Thus, there remains a need in the art to identify new agents and methods for preventing or treating cancer metastasis, including osteolytic bone metastases.

SUMMARY OF THE INVENTION

The materials and methods of the present invention fulfill the aforementioned and other related needs in the art. In one embodiment of the invention, a non-murine monoclonal antibody is provided, including functional fragment, that specifically binds to the same epitope of M-CSF as murine monoclonal antibody RX1 having the amino acid sequence set forth in Figure 4. In a related embodiment, an aformentioned antibody is provided wherein the antibody is selected from the group consisting of a polyclonal antibody; a monoclonal antibody; a humanized antibody; a human antibody; a chimeric antibody; Fab, F(ab')2; Fv; Sc Fv or SCA antibody fragment; a diabody; linear antibody; or a mutein of any one of these antibodies, that preferably retain binding affinity of at least 10^{-7}, 10^{-8} or 10^{-9} or higher. A non-murine monoclonal antibody, including functional fragment, that competes with monoclonal antibody RX1 having the amino acid sequence set forth in Figure 4 for binding to M-CSF by more than 75%.

In another embodiment, a non-murine monoclonal antibody, including functional fragment, wherein said non-murine monoclonal antibody or functional fragment thereof binds an epitope of M-CSF that includes at least 4, 5, 6, 7 or 8 contiguous residues of amino acids 98-105 of Figure 12 is provided.

In yet another embodiment, the aforementioned antibody or fragment that binds an epitope of M-CSF that includes amino acids 98-105 of Figure 12 is provided. In a related embodiment, the aforementioned antibody is provided comprising CDR3 of Figure 4A. In another embodiment, the antibody is provided comprising at least 1, 2, 3 , 4, 5, or 6 CDRs of murine antibody RX1 set forth in Figure 4A. In still another embodiment, the antibody comprisies at least 1, 2, 3, 4, or 5 CDRs of any of the 6 CDRs of antibody 5H4 set forth in Figure 16A-B. In another embodiment, the antibody comprises at least 1, 2, 3, 4, or 5 CDRs of any of the 6 CDRs of antibody MC1 set forth in Figure 16A-B. In yet another embodiment, the aforementioned antibody is provided comprising at least 1, 2, 3, 4, or 5 CDRs of any of the 6 CDRs of antibody MC3 set forth in Figure 16A-B. In a related embodiment, the antibody comprising at least 1, 2, 3, 4 or 5 CDRs of the consensus CDRs set forth in Figure 16A-B is provided. In still another related embodiment,the aforementioned antibody in which one or more residues of the consensus CDR(s) is substituted by the corresponding residue of any of the CDRs of antibody murine RX1, 5H4, MC1 or MC3 is provided. The desired binding affinity may be retained even though one or more of the amino acids in the antibody have been mutated, e.g. by conservative substitutions in the

CDRs, and/or conservative or non-conservative changes in the low and moderate risk residues.

In another embodiment of the inventionl, the aforementioned antibody is provided, comprising a variable heavy chain amino acid sequence which is at least 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% homologous to the amino acid sequence set forth in Figure 4A. In a related embodiment, the antibody comprises a variable light chain amino acid sequence which is at least 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% homologous to the amino acid sequence set forth in Figure 4A. In yet another embodiment, the antibody comprises a constant region and one or more heavy and light chain variable framework regions of a human antibody sequence.

In yet another embodiment of the invention, the aforementioned antibody is provided wherein the human antibody sequence is a human consensus sequence, human germline sequence, human consensus germline sequence, or any one of the human antibody sequences in Kabat, NCBI Ig Blast, http://www.ncbi.nlm.nih.gov/igblast/showGermline.cgi, Kabat Database http://www.bioinf.org.uk/abs/seqtest.html, FTP site for Kabat Release 5.0 (1992) ftp://ftp.ncbi.nih.gov/repository/kabat/Rel5.0/, ImMunoGeneTics database (Montpellier France) http://imgt.cnusc.fr:8104/, V-Base http://www.mrc-cpe.cam.ac.uk/LIST.php?menu=901, Zurich University http://www.unizh.ch/~antibody/Sequences/index.html, The Therapeutic Antibody Human Homology Project (TAHHP) http://www.path.cam.ac.uk/~mrc7/humanisation/TAHHP.html, Protein Sequence and Structure Analysis of Antibody Domains http://how.to/AnalyseAntibodies/, Humanization by design http://people.cryst.bbk.ac.uk/~ubcg07s/, Antibody Resources http://www.antibodyresource.com/educational.html, Antibody Engineering (by TT Wu), Humana Press. In still another embodiment, the aforementioned antibody is provided wherein the human antibody sequence is any one of the sequences set forth in Figures 23-24.

In another embodiment, the aforementioned antibody is provided, wherein the heavy chain variable region comprises the amino acid sequence $X_1VX_2LX_3EX_4GX_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}LX_{14}LX_{15}CX_{16}VX_{17}DYSITSDYAWNWIX_{18}QX_{19}X_{20}X_{21}X_{22}X_{23}LX_{24}WMGYISYSGSTSX_{25}NX_{26}X_{27}LX_{28}X_{29}X_{30}IX_{31}IX_{32}RX_{33}X_{34}X_{35}X_{36}X_{37}X_{38}FX_{39}LX_{40}LX_{41}X_{42}VX_{43}X_{44}X_{45}DX_{46}AX_{47}YYCASFDYAHAMDYWGX_{48}GTX_{49}VX_{50}VX_{51}X_{52}$, wherein X is any amino acid. In a related embodiment, the antibody is provided wherein the heavy chain variable region comprises the amino acid sequence

$DVX_1LX_2EX_3GPX_4X_5VX_6PX_7X_8X_9LX_{10}LX_{11}CX_{12}VTDYSITSDYAWNWIRQX_{13}PX_{14}X_{15}K$
$LEWMGYISYSGSTSYNPSLKX_{16}RIX_{17}IX_{18}RX_{19}TX_{20}X_{21}NX_{22}FX_{23}LX_{24}LX_{25}X_{26}VX_{27}X_{28}X$
$_{29}DX_{30}ATYYCASFDYAHAMDYWGX_{31}GTX_{32}VX_{33}VX_{34}X_{35}$, wherein X is any amino acid.

In still another embodiment of the invention, the aforementioned antibody is provided, wherein the heavy chain variable region comprises the amino acid sequence $X_1VQLQESGPGLVKPSQX_2LSLTCTVX_3DYSITSDYAWNWIRQFPGX_4X_5LEWMGYISY$
$SGSTSYNPSLKSRIX_6IX_7RDTSKNQFX_8LQLNSVTX_9X_{10}DTAX_{11}YYCASFDYAHAMDY$
$WGQGTX_{12}VTVSS$, wherein X is any amino acid. In a related embodiment, the antibody is provided, wherein the heavy chain variable region comprises the amino acid sequence
$DVQLQESGPGLVKPSQX_1LSLTCTVTDYSITSDYAWNWIRQFPGX_2KLEWMGYISYSG$
$STSYNPSLKSRIX_3IX_4RDTSKNQFX_5LQLNSVTX_6X_7DTATYYCASFDYAHAMDYWGQ$
GTX_8VTVSS, wherein X is any amino acid. In yet another embodiment, the antibody is provided wherein the heavy chain variable region comprises the amino acid sequence
DVQLQESGPGLVKPSQTLSLTCTVTDYSITSDYAWNWIRQFPGKKLEWMGYISYSGS
TSYNPSLKSRITISRDTSKNQFSLQLNSVTAADTATYYCASFDYAHAMDYWGQGTTV
TVSS. In still another embodiment, the antibody is provided wherein the heavy chain variable region comprises the amino acid sequence
QVQLQESGPGLVKPSQTLSLTCTVSDYSITSDYAWNWIRQFPGKGLEWMGYISYSGS
TSYNPSLKSRITISRDTSKNQFSLQLNSVTAADTAVYYCASFDYAHAMDYWGQGTT
VTVSS.

In another embodiment of the invention, the aforementioned antibody is provided wherein the light chain variable region comprises the amino acid sequence
$X_1IX_2LX_3QX_4X_5X_6X_7X_8X_9VX_{10}X_{11}X_{12}X_{13}X_{14}VX_{15}FX_{16}CX_{17}AX_{18}QSIGTSIHWYX_{19}QX_{20}X$
$_{21}X_{22}X_{23}X_{24}PX_{25}LLIKYASEX_{26}X_{27}X_{28}X_{29}IX_{30}X_{31}X_{32}FX_{33}GX_{34}GX_{35}GX_{36}X_{37}FX_{38}LX_{39}IX_{40}$
$X_{41}VX_{42}X_{43}X_{44}DX_{45}ADYYCQQINSWPTTFGX_{46}GTX_{47}LX_{48}X_{49}X_{50}X_{51}X_{52}$, wherein X is any amino acid. In a related embodiment, the antibody is provided wherein the light chain variable region comprises the amino acid sequence
$X_1IX_2LX_3QX_4PX_5X_6LX_7VX_8PX_9X_{10}X_{11}VX_{12}FX_{13}CX_{14}ASQSIGTSIHWYQQX_{15}TX_{16}X_{17}SP$
$RLLIKYASEX_{18}ISX_{19}IPX_{20}RFX_{21}GX_{22}GX_{23}GX_{24}X_{25}FX_{26}LX_{27}IX_{28}X_{29}VX_{30}X_{31}X_{32}DX_{33}AD$
$YYCQQINSWPTTFGX_{34}GTX_{35}LX_{36}X_{37}X_{38}X_{39}X_{40}$, wherein X is any amino acid. In yet another embodiment, the antibody is provided wherein the light chain variable region comprises the amino acid sequence
$X_1IX_2LTQSPX_3X_4LSVSPGERVX_5FSCRASQSIGTSIHWYQQX_6TX_7X_8X_9PRLLIKYASEX$

$_{10}X_{11}X_{12}GIPX_{13}RFSGSGSGTDFTLX_{14}IX_{15}X_{16}VESEDX_{17}ADYYCQQINSWPTTFGX_{18}GT$ KLEIKRX_{19}, wherein X is any amino acid.

In another embodiment of the invention, the aforementioned antibody is provided wherein the light chain variable region comprises the amino acid sequence $X_1IX_2LTQSPX_3X_4LSVSPGERVX_5FSCRASQSIGTSIHWYQQX_6TX_7X_8SPRLLIKYASEX_9I$ $SGIPX_{10}RFSGSGSGTDFTLX_{11}IX_{12}X_{13}VESEDX_{14}ADYYCQQINSWPTTFGX_{15}GTKLEIK$ RX_{16}, wherein X is any amino acid. In a related embodiment, the antibody is provided wherein the light chain variable region comprises the amino acid sequence $X_1IX_2LTQSPX_3X_4LSVSPGERVX_5FSCRASQSIGTSIHWYQQX_6TX_7X_8X_9PRLLIKYASESI$ $SGIPX_{10}RFSGSGSGTDFTLX_{11}IX_{12}X_{13}VESEDX_{14}ADYYCQQINSWPTTFGX_{15}GTKLEIK$ RX_{16}, wherein X is any amino acid. In yet another embodiment, the antibody is provided wherein the light chain variable region comprises the amino acid sequence EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIHWYQQKTGQAPRLLIKYASESISGIPD RFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPTTFGQGTKLEIKRT.

In still another embodiment of the invention, the aforementioned antibody is provided wherein the light chain variable region comprises the amino acid sequence EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIHWYQQKTGQAPRLLIKYASERATGIP DRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPTTFGQGTKLEIKRT. In another embodiment, the antibody is provided, wherein the light chain variable region comprises the amino acid sequence EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIHWYQQKTGQSPRLLIKYASERISGIPD RFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPTTFGQGTKLEIKRT.

Due to the three-dimensional structural role of prolines, modifications at prolines are generally considered to be at least moderate risk changes, even if the position is typically a low risk position. Subtitutional changes are preferred but insertions and deletions are also possible.

In another embodiment of the invention, the aforementioned antibody wherein at least one X is a corresponding amino acid within the amino acid sequence set forth in Figure 4A is provided. In a related embodiment, the antibody is provided wherein at least one X is a conservative substitution (according to Table 1) of a corresponding amino acid within the amino acid sequence set forth in Figure 4A. In yet another related embodiment, the antibody is provided wherein at least one X is a non-conservative substitution (according

to Table 1) of a corresponding amino acid within the amino acid sequence set forth in Figure 4A. In still another embodiment of the invention, the antibody is provided wherein at least one X is a corresponding amino acid within a human antibody sequence. In addition the antibody comprises the human antibody consensus sequence, human germline sequence, human consensus germline sequence, or any one of the human antibody sequences in Kabat, NCBI Ig Blast, http://www.ncbi.nlm.nih.gov/igblast/showGermline.cgi, Kabat Database http://www.bioinf.org.uk/abs/seqtest.html, FTP site for Kabat Release 5.0 (1992) ftp://ftp.ncbi.nih.gov/repository/kabat/Rel5.0/, ImMunoGeneTics database (Montpellier France) http://imgt.cnusc.fr:8104/, V-Base http://www.mrc-cpe.cam.ac.uk/LIST.php?menu=901, Zurich University http://www.unizh.ch/~antibody/Sequences/index.html, The Therapeutic Antibody Human Homology Project (TAHHP) http://www.path.cam.ac.uk/~mrc7/humanisation/TAHHP.html, Protein Sequence and Structure Analysis of Antibody Domains http://how.to/AnalyseAntibodies/, Humanization by design http://people.cryst.bbk.ac.uk/~ubcg07s/, Antibody Resources http://www.antibodyresource.com/educational.html, Antibody Engineering (by TT Wu), Humana Press.

In another embodiment of the invention, the aforementioned antibody wherein the human antibody sequence is one of the sequences set forth in Figures 23-24 is provided. In another embodiment, the antibody comprises a variable heavy chain amino acid sequence which is at least 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical to one of the heavy chain amino acid sequences set forth in Figure 19B. In yet another embodiment, the antibody comprises a variable light chain amino acid sequence which is at least 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical to one of the light chain amino acid sequences set forth in any of Figures 20B-22B.

In still another embodiment, an antibody is provided comprising a heavy chain as set forth above and a light chain as set forth above.

In yet another embodiment of the invention, the aforementioned antibody has an affinity Kd of at least 10[-7]. In a related embodiment, the antibody has an affinity Kd of at least 10[-9].

In another embodiment of the invention, the aforementioned antibody is provided which is a polyclonal antibody; a monoclonal antibody; a humanized antibody; a

human antibody; a chimeric antibody; Fab, F(ab')2, Fv, ScFv or SCA antibody fragment; a diabody; a linear antibody; or a mutein of any one of these antibodies. In a related embodiment, the monoclonal antibody is an isolated antibody.

In still another embodiment of the invention, an isolated nucleic acid is provded comprising a nucleic acid sequence encoding a light chain of the aforementioned antibody. In a related embodiment, the isolated nucleic acid comprises a heavy chain nucleic acid sequence which is at least 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical to the heavy chain nucleotide sequence set forth in Figure 4A. In yet another related embodiment, the isolated nucleic acid comprises a light chain nucleic acid sequence which is at least 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical to the light chain nucleotide sequence set forth in Figure 4A.

In another embodiment, a vector comprising the aforementioned isolated nucleic acid is provided. In a related embodiment, the aforementioned vector is provided wherein the isolated nucleic acid is operably linked to a regulatory control sequence. In still another embodiment, a host cell is provided comprising the aforementioned vector.

Numerous methods are contemplated in the present invention. For example, a method of producing an aforementioned antibody is provided comprising culturing the aforementioend host cell such that the isolated nucleic acid is expressed to produce the antibody. In a related embodiment, the method further comprises the step of recovering the antibody from the host cell culture. In a related embodiment, an isolated antibody produced by the aforementioned method is provided.

A hybridoma that secretes an aforementioned antibody is alos provded by the present invention. Additionally, an aforementioned antibody that is conjugated to a toxin is provided.

In another embodiment of the invention, a pharmaceutical composition is provided comprising any one of the aforementioned antibodies and a pharmaceutically suitable carrier, excipient or diluent. In a related embodiment, the pharmaceutical composition further comprises a second therapeutic agent. In yet another related embodiment, the pharmaceutical composition is provided wherein the second therapeutic agent is a cancer chemotherapeutic agent. In still another related embodiment, the pharmaceutical composition is provided wherein the second therapeutic agent is a bisphosphonate. In another embodiment the second therapeutic agent is another antibody.

Antibodies of the present invention are contemplated to have numerous desirable characteristics for the treatment of diseases and disorders. In one embodiment of the ivention, the any one of aforementioned antibodies that binds to M-CSF for preventing a subject afflicted with a disease that causes or contributes to osteolysis, wherein the antibody effectively reduces the severity of bone loss associated with the disease, is provided. Similarly, any one of aforementioned antibodies that binds to M-CSF is provided for treating a subject afflicted with a disease that causes or contributes to osteolysis, wherein said antibody effectively reduces the severity of bone loss associated with the disease.

Numerous diseases and disorders are contmeplted to be amenable to antibody-based treatment in the present invention. In one embodiment of the invention, the aforementioned antibody is provided wherein said disease is selected from the group consisting of metabolic bone diseases associated with relatively increased osteoclast activity, including endocrinopathies (hypercortisolism, hypogonadism, primary or secondary hyperparathyroidism, hyperthyroidism), hypercalcemia, deficiency states (rickets/osteomalacia, scurvy, malnutrition), chronic diseases (malabsorption syndromes, chronic renal failure (renal osteodystrophy), chronic liver disease (hepatic osteodystrophy)), drugs (glucocorticoids (glucocorticoid-induced osteoporosis), heparin, alcohol), and hereditary diseases (osteogenesis imperfecta, homocystinuria), cancer, osteoporosis, osteopetrosis, inflammation of bone associated with arthritis and rheumatoid arthritis, periodontal disease, fibrous dysplasia, and/or Paget's disease.

In a related embodiment, the aforementioned antibody that binds to M-CSFis provided for preventing or treating metastatic cancer to bone, wherein the metastatic cancer is breast, lung, renal, multiple myeloma, thyroid, prostate, adenocarcinoma, blood cell malignancies, including leukemia and lymphoma; head and neck cancers; gastrointestinal cancers, including esophageal cancer, stomach cancer, colon cancer, intestinal cancer, colorectal cancer, rectal cancer, pancreatic cancer, liver cancer, cancer of the bile duct or gall bladder; malignancies of the female genital tract, including ovarian carcinoma, uterine endometrial cancers, vaginal cancer, and cervical cancer; bladder cancer; brain cancer, including neuroblastoma; sarcoma, osteosarcoma; and skin cancer, including malignant melanoma or squamous cell cancer.

In another embodiment of the invention, a method of screening for an M-CSF-specific antibody is provided comprising the steps of contacting metastatic tumor cell medium, osteoclasts and a candidate antibody; detecting osteoclast formation, proliferation

and/or differentiation; and identifying said candidate antibody as an M-CSF-specific antibody if a decrease in osteoclast formation, proliferation and/or differentiation is detected. Similarly, the aforementioned method is provided wherein said metastatic tumor cell medium includes tumor cells.

In another embodiment, the aformentioned method is provided wherein the contacting step (a) occurs in vivo, said detecting step (b) comprises detecting size and/or number of bone metastases, and the candidate antibody is identified as an M-CSF-specific antibody if a decrease in size and/or number of bone metastases is detected. In a related embodiment, the aforementioned method is provided further comprising the step of determining if the candidate antibody binds to M-CSF. Similarly, another embodiment of the invention provides the aforementioned method further comprising the step of determining if said candidate antibody inhibits interaction between M-CSF and its receptor M-CSFR.

In another embodiment of the invention, a method of identifying an M-CSF-specific antibody that can prevent or treat metastatic cancer to bone is provided, comprising the steps of: (a) detecting binding of a candidate antibody to an epitope of M-CSF that includes at least 4 contiguous residues of amino acids 98-105 of Figure 12; and (b) assaying the ability of said candidate antibody to prevent or treat metastatic cancer to bone *in vitro* or *in vivo*.

In yet another embodiment of the invention, a method of altering a CDR of an antibody that binds an epitope of M-CSF that includes amino acids 98-105 of Figure 12 is provided comprising altering an amino acid within a CDR of the amino acid sequence set forth in Figure 4A and selecting for an antibody that binds M-CSF with an affinity Ka of at least 10[-7]. In another embodiment, a method of systematically altering up to 60% of the heavy chain amino acid sequence set forth in Figure 4A is provided comprising altering any of X1-X52 in the amino acid sequence $X_1VX_2LX_3EX_4GX_5X_6X_7X_8X_9X_{10}X_{11}X_{12}X_{13}LX_{14}LX_{15}CX_{16}VX_{17}DYSITSDYAWNWIX_{18}QX_{19}X_{20}X_{21}X_{22}X_{23}LX_{24}WMGYISYSGSTSX_{25}NX_{26}X_{27}LX_{28}X_{29}X_{30}IX_{31}IX_{32}RX_{33}X_{34}X_{35}X_{36}X_{37}X_{38}FX_{39}LX_{40}LX_{41}X_{42}VX_{43}X_{44}X_{45}DX_{46}AX_{47}YYCASFDYAHAMDYWGX_{48}GTX_{49}VX_{50}VX_{51}X_{52}$, and testing an antibody comprising the altered amino acid sequence for binding to an epitope of M-CSF that includes amino acids 98-105 of Figure 12.

In a related embodiment, a method of systematically altering up to 60% of the light chain amino acid sequence set forth in Figure 4A is provided comprising altering any of

X1-X52 in the amino acid sequence $X_1IX_2LX_3QX_4X_5X_6X_7X_8X_9VX_{10}X_{11}X_{12}X_{13}X_{14}VX_{15}FX_{16}CX_{17}AX_{18}$QSIGTSIHWY$X_{19}QX_{20}X_{21}X_{22}X_{23}X_{24}PX_{25}$LLIKYASE$X_{26}X_{27}X_{28}X_{29}IX_{30}X_{31}X_{32}FX_{33}GX_{34}GX_{35}GX_{36}X_{37}FX_{38}LX_{39}IX_{40}X_{41}VX_{42}X_{43}X_{44}DX_{45}$ADYYCQQINSWPTTFG$X_{46}GTX_{47}LX_{48}X_{49}X_{50}X_{51}X_{52}$, and testing an antibody comprising the altered amino acid sequence for binding to an epitope of M-CSF that includes amino acids 98-105 of Figure 12.

In another embodiment of the invention, a method of expressing an antibody having CDRs designed by the aforementioned process is provided. In another embodiment, a pharmaceutical composition comprising an antibody that binds MCSF wherein said antibody is made using the aforementioned method is provided.

In still another embodiment of the invention, a method of preventing or reducing bone loss is provided comprising administering to a subject afflicted with a disease that causes or contributes to osteolysis a therapeutically effective amount of any one of the aforementioned antibodies, thereby preventing or reducing bone loss associated with the disease. In a related embodiment, a method of treating a subject afflicted with a disease that causes or contributes to osteolysis is provided comprising administering to said subject a therapeutically effective amount of the antibody of any one of the aforementioned antibodies, thereby reducing the severity of bone loss associated with the disease.

In a related embodiment, the aforementioned method is provided wherein said disease is selected from the group consisting of metabolic bone diseases associated with relatively increased osteoclast activity, including endocrinopathies (hypercortisolism, hypogonadism, primary or secondary hyperparathyroidism, hyperthyroidism), hypercalcemia, deficiency states (rickets/osteomalacia, scurvy, malnutrition), chronic diseases (malabsorption syndromes, chronic renal failure (renal osteodystrophy), chronic liver disease (hepatic osteodystrophy)), drugs (glucocorticoids (glucocorticoid-induced osteoporosis), heparin, alcohol), and hereditary diseases (osteogenesis imperfecta, homocystinuria), cancer, osteoporosis, osteopetrosis, inflammation of bone associated with arthritis and rheumatoid arthritis, periodontal disease, fibrous dysplasia, and/or Paget's disease.

In still another embodiment, a method of preventing or treating metastatic cancer to bone is provided comprising administering to a subject afflicted with metastatic cancer a therapeutically effective amount of any one of the aforementioned antibodies. In a related embodiment, the method is provided wherein the metastatic cancer is breast, lung,

renal, multiple myeloma, thyroid, prostate, adenocarcinoma, blood cell malignancies, including leukemia and lymphoma; head and neck cancers; gastrointestinal cancers, including esophageal cancer, stomach cancer, colon cancer, intestinal cancer, colorectal cancer, rectal cancer, pancreatic cancer, liver cancer, cancer of the bile duct or gall bladder; malignancies of the female genital tract, including ovarian carcinoma, uterine endometrial cancers, vaginal cancer, and cervical cancer; bladder cancer; brain cancer, including neuroblastoma; sarcoma, osteosarcoma; and skin cancer, including malignant melanoma or squamous cell cancer.

In yet another embodiment of the invention, a method of preventing bone loss and tumor growth is provded comprising administering to a subject in need thereof therapeutically effective amounts of any one of the aforementioned antibodies. In a related embodiment, the method further comprises administering a second therapeutic agent. In still another related embodiment, the method is provided wherein the second therapeutic agent is a cancer chemotherapeutic agent or a bisphosphonate. In yet another related embodiment, the method is provided wherein the bisphonate is zeledronate, pamidronate, clodronate, etidronate, tilundronate, alendronate, or ibandronate. In yet another related embodiment, the aforementioned methods are provided wherein the therapeutic agent is a cytotoxic chemotherapeutic agent. In another embodiment, the aforementioned method is provided wherein the subject is precluded from receiving bisphophonate treatment.

In still another related embodiment, the aforementioned method is provided wherein the antibody is effective to reduce the dosage of second therapeutic agent required to achieve a therapeutic effect. In another embodiment, the second therapeutic agent is a non-M-CSF colony stimulating factor, for example G-CSF.

In another embodiment of the invention, the aforementioned methods are provided wherein the subject is a mammal. In a related embodiment, the mammal is human.

In another embodiment, the aforementioned methods are provided wherein the antibody inhibits the interaction between M-CSF and its receptor (M-CSFR). In another related embodinment, the antibody inhibits osteoclast proliferation and/or differentiation induced by tumor cells. In yet another embodiment, the aforementioned methods are provided wherein the antibody is administered at a dose between about 2 μg/kg to 30 mg/kg, 0.1 mg/kg to 30 mg/kg or 0.1 mg/kg to 10 mg/kg body weight.

In still another embodiment of the invention, a package, vial or container is provided comprising a medicament comprising one or more of the aforementioned antibodies

and instructions that the medicament should be used in combination with surgery or radiation therapy. In another embodiment, a method of preventing or treating metastatic cancer to bone comprising the steps of administering any one of the aforementioned antibodies to a subject and treating the subject with surgery or radiation therapy is provided. In another embodiment, a method of targeting a tumor cell expressing membrane-bound M-CSF on its surface is provided comprising the step of administering any one of the aforementioned antibodies, wherein the antibody is conjugated to a radionuclide or other toxin. In yet another embodiment, a method of treating a subject suffering from a cancer is provided comprising administering a therapeutically effective amount of the any one of the aforementioned antibodies.

In still another embodiment of the invention, a method of preventing bone loss is provided comprising administering to a subject afflicted with a disease that causes or contributes to osteolysis an amount of any one of the aforementioned antibodies in an amount effective to neutralize M-CSF produced by the subject's cells, the amount being larger than the amount effective to neutralize M-CSF produced by the cancer cells. In a related embodiment, a method of treating a subject afflicted with a disease that causes or contributes to osteolysis is provided comprising administering to said subject an amount of any one of the aforementioned antibodies in an amount effective to neutralize M-CSF produced by the subject's cells, the amount being larger than the amount effective to neutralize M-CSF produced by the cancer cells.

In one embodiment of the invention, a pharmaceutical composition is provided comprising antibody RX1, or a non-murine RX-1 derived antibody or an RX-1 competing antibody, and a cancer therapeutic agent. In another embodiment of the invention, a package, vial or container is provided comprising a medicament comprising antibody RX1, or a non-murine RX-1 derived antibody or an RX-1 competing antibody, and instructions that the medicament should be used in combination with surgery or radiation therapy.

In still another embodiment of the invention, a method of treating a subject suffering from a cancer is provided, wherein the cells comprising the cancer do not secrete M-CSF, comprising the step of administering any one of the aforementioned antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a topology diagram showing the disulfide bonds in truncated dimeric M-CSF.

Figure 2 is a stereodiagram of the C-alpha backbone of M-CSF with every tenth residue labeled and with the non-crystallographic symmetry axis indicated by a dotted line.

Figure 3 is a comparison of osteoclast inducing activity between purified M-CSF and conditioned medium (CM) from MDA 231 cells and MCF7 cells.

Figure 4A shows the amino acid sequence of M-CSF-specific murine antibody RX1 and a corresponding nucleic acid sequence. The CDR regions are numbered and shown in bold.

Figures 4B and 4C show the amino acid sequences of M-CSF specific murine antibody RX1 light and heavy chains, respectively, with high risk (bold), moderate risk (underline), and low risk residues identified according to Studnicka et al., WO93/11794.

Figure 5 shows that M-CSF antibodies RX1 and 5A1 are species specific.

Figure 6 shows that that antibody RX1 effectively inhibits osteolysis in a human xenograft model at a concentration 5mg/kg.

Figure 7 shows that the number of metastases is reduced when antibody RX1 is administered to human breast cancer MDA-MB-231 bearing nude mice at a concentration of 5 mg/kg.

Figure 8A and 8B shows that an M-CSF-specific antibody bound to breast cancer cell line MDA-MB-231 or to multiple myeloma cancer cell line ARH77.

Figure 9 shows that M-CSF is prevalent on a number of cancer cell surfaces.

Figure 10 is the amino acid sequence of M-CSFα.

Figure 11 is is the amino acid sequence of M-CSFβ.

Figure 12 is is the amino acid sequence of M-CSFγ.

Figures 13, 14, and 15 show the amino acid sequences of MCSF-specific murine antibodies 5H4, MC1 and MC3, respectively.

Figures 16A and B are an alignment of CDR regions of the heavy and light

chain amino acid sequences of human M-CSF specific murine antibodies RX1; 5H4; MC1; and MC3.

Figure 17 shows the neutralization activities of intact versus Fab fragments for RX1 versus 5H4.

Figure 18 shows the structure of M-CSF with RX1, 5H4, and MC3 epitopes highlighted.

Figure 19A shows (a) the risk line for the murine RX1 heavy chain (H=high risk, M=moderate risk, L=low risk), (b) the RX1 heavy chain amino acid sequence, (c) the amino acid sequence of the closest human consensus sequence, Kabat Vh2 consensus, aligned to RX1 and (d) changes that were made to produce two exemplary human engineered sequences. Figure 19B shows the amino acid sequences of the two exemplary heavy chain human engineered sequences, designated "low risk" and "low+moderate risk" as well as corresponding nucleic acid sequences.

Figure 20A shows (a) the risk line for the murine RX1 light chain (H=high risk, M=moderate risk, L=low risk), (b) the RX1 light chain amino acid sequence, (c) the amino acid sequence of the closest human consensus sequence, Kabat Vk3 consensus, aligned to RX1 and (d) changes that were made to produce two exemplary human engineered sequences. Figure 20B shows the amino acid sequences of the two exemplary light chain human engineered sequences, designated "low risk" and "low+moderate risk" as well as corresponding nucleic acid sequences.

Figure 21A shows (a) the risk line for the murine RX1 light chain (H=high risk, M=moderate risk, L=low risk), (b) the RX1 light chain amino acid sequence, (c) the amino acid sequence of the closest human consensus sequence, Kabat Vk3 consensus, aligned to RX1 and (d) an alternate exemplary amino acid sequence in which positions 54-56 were not changed (i.e. remained the murine sequence). Figure 21B shows the amino acid sequences of two exemplary alternate light chain human engineered sequences, designated "alternate low risk" and "alternate low+moderate risk" as well as corresponding nucleic acid sequences.

Figure 22A shows (a) the risk line for the murine RX1 light chain (H=high risk, M=moderate risk, L=low risk), (b) the RX1 light chain amino acid sequence, (c) the amino acid sequence of the closest human consensus germline sequence, Vk6 Subgroup 2-1-(1) A14, aligned to RX1 and (d) changes that were made to produce two exemplary human

engineered sequences. Figure 22B shows the amino acid sequences of the two exemplary light chain human engineered sequences, designated "low risk" and "low+moderate risk" as well as corresponding nucleic acid sequences.

Figures 23A and 23B show the alignment of murine RX1 light chain amino acid sequence with various human consensus and human germline consensus sequences using the Kabat numbering system (amino acid numbering indicated in line designated "POS").

Figures 24A and 24B show the alignment of murine RX1 heavy chain amino acid sequence with various human consensus and human germline consensus sequences using the Kabat numbering system (amino acid numbering indicated in line designated "POS").

DETAILED DESCRIPTION

The ability to metastasize is a defining characteristic of a cancer. Metastasis refers to the spread of cancer cells to other parts of the body or to the condition produced by this spread. Metastasis is a complex multi-step process that includes changes in the genetic material of a cell, uncontrolled proliferation of the altered cell to form a primary tumor, development of a new blood supply for the primary tumor, invasion of the circulatory system by cells from the primary tumor, dispersal of small clumps of primary tumor cells to other parts of the body, and the growth of secondary tumors in those sites.

Bone is one of the most common sites of metastasis in human breast, lung, prostate and thyroid cancers, as well as other cancers, and in autopsies as many as 60% of cancer patients are found to have bone metastasis. Osteolytic bone metastasis shows a unique step of osteoclastic bone resorption that is not seen in metastasis to other organs. Bone loss associated with cancer metastasis is mediated by osteoclasts (multinucleated giant cells with the capacity to resorb mineralized tissues), which seem to be activated by tumor products.

Colony stimulating factor (CSF 1), also known as macrophage colony stimulating factor (M-CSF), has been found crucial for osteoclast formation. In addition, M-CSF has been shown to modulate the osteoclastic functions of mature osteoclasts, their migration and their survival in cooperation with other soluble factors and cell to cell interactions provided by osteoblasts and fibroblasts (Fixe and Praloran, Cytokine 10: 3-7, 1998; Martin et al., Critical Rev. in Eukaryotic Gene Expression 8: 107-23 (1998)).

The full-length human M-CSF mRNA encodes a precursor protein of 554

amino acids. Through alternative mRNA splicing and differential post-translational proteolytic processing, M-CSF can either be secreted into the circulation as a glycoprotein or chondroitin sulfate containing proteoglycan or be expressed as a membrane spanning glycoprotein on the surface of M-CSF producing cells. The three-dimensional structure of the bacterially expressed amino terminal 150 amino acids of human M-CSF, the minimal sequence required for full in vitro biological activity, indicates that this protein is a disulfide linked dimer with each monomer consisting of four alpha helical bundles and an anti-parallel beta sheet (Pandit et al., Science 258: 1358-62 (1992)). Three distinct M-CSF species are produced through alternative mRNA splicing. The three polypeptide precursors are M-CFSα of 256 amino acids, M-CSFβ of 554 amino acids, and M-CSFγ of 438 amino acids. M-CSFβ is a secreted protein that does not occur in a membrane-bound form. M-CSFα is expressed as an integral membrane protein that is slowly released by proteolytic cleavage. M-CSFα is cleaved at amino acids 191-197 of the sequence set out in Figure 10. The membrane-bound form of M-CSF can interact with receptors on nearby cells and therefore mediates specific cell-to-cell contacts. The term "M-CSF" may also inlcude amino acids 36-438 of Figure 12.

Various forms of M-CSF function by binding to its receptor M-CSFR on target cells. M-CSFR is a membrane spanning molecule with five extracellular immunoglobulin-like domains, a transmembrane domain and an intracellular interrupted Src related tyrosine kinase domain. M-CSFR is encoded by the *c-fms* proto-oncogene. Binding of M-CSF to the extracellular domain of M-CSFR leads to dimerization of the receptor, which activates the cytoplasmic kinase domain, leading to autophosphorylation and phosphorylation of other cellular proteins (Hamilton J. A., J Leukoc Biol.,62(2):145-55 (1997); Hamilton J, A., Immuno Today., 18(7): 313-7(1997).

Phosphorylated cellular proteins induce a cascade of biochemical events leading to cellular responses: mitosis, secretion of cytokines, membrane ruffling, and regulation of transcription of its own receptor (Fixe and Praloran, Cytokine 10: 32-37 (1998)).

M-CSF is expressed in stromal cells, osteoblasts, and other cells. It is also expressed in breast, uterine, and ovarian tumor cells. The extent of expression in these tumors correlates with high grade and poor prognosis (Kacinski Ann. Med. 27: 79-85 (1995); Smith et al., Clin. Cancer Res. 1: 313-25 (1995)). In breast carcinomas, M-CSF expression is prevalent in invasive tumor cells as opposed to the intraductal (pre-invasive) cancer (Scholl et al., J. Natl. Cancer Inst. 86: 120-6 (1994)). In addition, M-CSF is shown to promote

progression of mammary tumors to malignancy (Lin et al., J. Exp. Med. 93: 727-39 (2001)). For breast and ovarian cancer, the production of M-CSF seems to be responsible for the recruitment of macrophages to the tumor.

There exists no report of using a M-CSF antibody in preventing or treating cancer metastasis or bone loss associated with cancer metastasis. It has been discovered, as part of the present invention, that an M-CSF-specific antibody neutralizes osteoclast induction by metastatic cancer cells and/or reduces metastases to bone in animal models of cancer. Thus, the present invention provides compositions and methods for treating or preventing cancer, cancer metastasis and bone loss associated with cancer metastasis.

A preferred anti-M-CSF antibody murine RX1 was modified to be less immunogenic in humans based on the human engineering method of Studnicka et al. In a preferred embodiment, 8 to 12 surface exposed amino acid residues of the heavy chain variable region and 16 to 19 surface exposed residues in the light chain region were modified to human residues in positions determined to be unlikely to adversely effect either antigen binding or protein folding, while reducing its immunogenicity with respect to a human environment. Synthetic genes containing modified heavy and/or light chain variable regions were constructed and linked to human γ heavy chain and/or kappa light chain constant regions. Any human heavy chain and light chain constant regions may be used in combination with the human engineered antibody variable regions. The human heavy and light chain genes were introduced into mammalian cells and the resultant recombinant immunoglobulin products were obtained and characterized.

The term "RX 1-derived antibody" includes any one of the following:

1) an amino acid variant of murine antibody RX1 having the amino acid sequence set out in Figure 4, including variants comprising a variable heavy chain amino acid sequence which is at least 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% homologous to the amino acid sequence as set forth in Figure 4 and/or comprising a variable light chain amino acid sequence which is at least 60, 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% homologous to the amino acid sequence as set forth in Figure 4, taking into account similar amino acids for the homology determination;

2) MCSF-binding polypeptides (excluding murine antibody RX1) comprising one or more complementary determining regions (CDRs) of murine antibody RX1 having the amino acid sequence set out in Figure 4, preferably comprising at least

CDR3 of the RX1 heavy chain, and preferably comprising two or more, or three or more, or four or more, or five or more, or all six CDRs;

3) human engineered antibodies having the heavy and light chain amino acid sequences set out in Figures 19B through 22B or variants thereof comprising a heavy or light chain having at least 60% amino acid sequence identity with the original human engineered heavy or the light chain of Figures 19B through 22B, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%, including for example, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100% identical;

4) MCSF-binding polypeptides (excluding murine antibody RX1) comprising the high risk residues of one or more CDRs of the human engineered antibodies of Figures 19B through 22B, and preferably comprising high risk residues of two or more, or three or more, or four or more, or five or more, or all six CDRs;

5) human engineered antibodies or variants retaining the high risk amino acid residues set out in Figure 4B, and comprising one or more changes at the low or moderate risk residues set out in Figure 4B;

for example, comprising one or more changes at a low risk residue and conservative substitutions at a moderate risk residue set out in Figure 4B, or

for example, retaining the moderate and high risk amino acid residues set out in Figure 4B and comprising one or more changes at a low risk residue,

where changes include insertions, deletions or substitutions and may be conservative substitutions or may cause the engineered antibody to be closer in sequence to a human light chain or heavy chain sequence, a human germline light chain or heavy chain sequence, a consensus human light chain or heavy chain sequence, or a consensus human germline light chain or heavy chain sequence;

that retain ability to bind M-CSF. Such antibodies preferably bind to M-CSF with an affinity of at least 10^{-7}, 10^{-8} or 10^{-9} or higher and preferably neutralize the osteoclastogenesis inducing activity of M-CSF. Optionally, any chimeric, human or humanized M-CSF antibody publicly disclosed before the filing date hereof, or disclosed in an application filed before the filing date hereof, is excluded from the scope of the invention.

The term "RX 1-competing antibody" includes

1) a non-murine or non-rodent monoclonal antibody that binds to the same epitope of M-CSF as murine RX1 having the complete light and heavy chain sequences set out in Figure 4;

2) a non-murine or non-rodent monoclonal antibody that binds to at least 4 contiguous amino acids of amino acids 98-105 of the M-CSF of Figure12; and

3) a non-murine or non-rodent monoclonal antibody that competes with murine antibody RX1 having the complete sequence set out in Figure 4 for binding to M-CSF, by more than 75%, more than 80%, or more than 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94% or 95%. Such antibodies preferably bind to M-CSF with an affinity of at least 10^{-7}, 10^{-8} or 10^{-9} or higher and preferably neutralize the osteoclastogenesis inducing activity of M-CSF. Optionally, any chimeric, human or humanized M-CSF antibody publicly disclosed before the filing date hereof, or disclosed in an application filed before the filing date hereof, is excluded from the scope of the invention.

"Non-rodent" monoclonal antibody is any antibody, as broadly defined herein, that is not a complete intact rodent monoclonal antibody generated by a rodent hybridoma. Thus, non-rodent antibodies specifically include, but are not limited to, variants of rodent antibodies, rodent antibody fragments, linear antibodies, chimeric antibodies, humanized antibodies, and human antibodies, including human antibodies produced from transgenic animals or via phage display technology. Similarly, non-murine antibodies include but are not limited to variants of murine antibodies, murine antibody fragments, linear antibodies, chimeric, humanized and human antibodies.

"Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma,; lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include breast cancer, prostate cancer, colon cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

"Treatment" is an intervention performed with the intention of preventing the development or altering the pathology of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. In tumor (e.g., cancer) treatment, a therapeutic agent may directly decrease the pathology of tumor cells, or render the tumor cells more susceptible to treatment by other therapeutic agents, e.g., radiation and/or chemotherapy. Treatment may include alleviating clinical, biochemical, radiological or subjective symptoms of the disease, or reducing the predisposition to the disease. The "pathology" of cancer includes all phenomena that compromise the well being of the patient. This includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, etc. Thus, improvement after treatment may be manifested as decreased tumor size, decline in tumor growth rate, destruction of existing tumor cells or metastatic cells, and/or a reduction in the size or number of metastases.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

As used herein, the phrase "metastatic cancer" is defined as cancers that have potential to spread to other areas of the body, particularly bone. A variety of cancers can metastasize to the bone, but the most common metastasizing cancers are breast, lung, renal, multiple myeloma, thyroid and prostate. By way of example, other cancers that have the potential to metastasize to bone include but are not limited to adenocarcinoma, blood cell malignancies, including leukemia and lymphoma; head and neck cancers; gastrointestinal cancers, including esophageal cancer, stomach cancer, colon cancer, intestinal cancer, colorectal cancer, rectal cancer, pancreatic cancer, liver cancer, cancer of the bile duct or gall bladder; malignancies of the female genital tract, including ovarian carcinoma, uterine endometrial cancers, vaginal cancer, and cervical cancer; bladder cancer; brain cancer, including neuroblastoma; sarcoma, osteosarcoma; and skin cancer, including malignant melanoma and squamous cell cancer. The present invention especially contemplates prevention and treatment of tumor-induced osteolytic lesions in bone.

As used herein, the phrase "therapeutically effective amount" refers to is

meant to refer to an amount of therapeutic or prophylactic M-CSF antibody that would be appropriate for an embodiment of the present invention, that will elicit the desired therapeutic or prophylactic effect or response when administered in accordance with the desired treatment regimen.

Human "M-CSF" as used herein refers to a human polypeptide having substantially the same amino acid sequence as the mature human M-CSFα, M-CSFβ, or M-CSFγ polypeptides described in Kawasaki et al., Science 230:291 (1985), Cerretti et al., Molecular Immunology, 25:761 (1988), or Ladner et al., EMBO Journal 6:2693 (1987), each of which are incorporated herein by reference. Such terminology reflects the understanding that the three mature M-CSFs have different amino acid sequences, as described above, and that the active form of M-CSF is a disulfide bonded dimer; thus, when the term "M-CSF" refers to the biologically active form, the dimeric form is intended. "M-CSF dimer" refers to two M-CSF polypeptide monomers that have dimerized and includes both homodimers (consisting of two of the same type of M-CSF monomer) and heterodimers (consisting of two different monomers). M-CSF monomers may be converted to M-CSF dimers in vitro as described in U.S. Pat. No. 4,929,700, which is incorporated herein by reference.

Anti-MCSF antibodies

The present invention provides a M-CSF-specific antibody, such as RX1, 5H4, MC1, and/or MC3, pharmaceutical formulations including a M-CSF-specific antibody, such as RX1, 5H4, MC1, and/or MC3, methods of preparing the pharmaceutical formulations, and methods of treating patients with the pharmaceutical formulations and compounds. The term "antibody" is used in the broadest sense and includes fully assembled antibodies, monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), antibody fragments that can bind antigen (e.g., Fab', F'(ab)2, Fv, single chain antibodies, diabodies), and recombinant peptides comprising the forgoing as long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that are typically include different antibodies directed against different

determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the homogeneous culture, uncontaminated by other immunoglobulins with different specificities and characteristics.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., Nature, 256:495 [19751, or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described inClackson et al.,Nature,352:624628[1991] end Marks et al., J. Mol. Biol., 222:581-597 (1991), for example.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes, IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses or isotypes, e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma and mu respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. Different isotypes have different effector functions; for example, IgG1 and IgG3 isotypes have ADCC activity.

"Antibody fragments" comprise a portion of an intact full length antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies (Zapata et al., Protein Eng.,8(10):1057-1062 (1995)); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize 35 readily. Pepsin treatment yields an F(ab')2 fragment that has two "Single-chain Fv" or "sFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains that enables the Fv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in The

Pharmacology of Monoclonal Antibodies, vol. 1 13, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "hypervariable" region refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a complementarity determining region or CDR [i.e., residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain as described by Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)] and/or those residues from a hypervariable loop (*i.e.*, residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain as described by [Chothia et al., *J. Mol.Biol. 196*: 901-917 (1987)].

"Framework" or FR residues are those variable domain residues other than the hypervariable region residues.

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and 30 Hollinger et al., Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

In some embodiments, it may be desirable to generate multispecific (e.g. bispecific) humanized or variant anti-M-CSF antibodies having binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of M-CSF. Alternatively, an anti-M-CSF arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g., CD2 or CD3), or Fc receptors for IgG (FcγR), such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the M-CSF-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express M-CSF. These antibodies possess an M-CSF-binding arm and an arm which binds the cytotoxic agent (e.g., saporin, anti-interferon-60, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody

fragments (e.g., F(ab').sub.2 bispecific antibodies).

According to another approach for making bispecific antibodies, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. See WO96/27011 published Sep. 6, 1996.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., Science 229:81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes. In yet a further embodiment, Fab'-SH fragments directly recovered from E. coli can be chemically coupled in vitro to form bispecific antibodies. (Shalaby et al., J. Exp. Med. 175:217-225 (1992))

Shalaby et al., J. Exp. Med. 175:217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E.coli* and subjected to directed chemical coupling in vitro to form the

bispecfic antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. (Kostelny et al., J. Immunol. 148(5):1547-1553 (1992)) The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., Proc. Natl. Acad. Sci. USA 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments.

The fragments comprise a heavy chain variable region (V_H) connected to a light-chain variable region (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. *See* Gruber et al., J. Immunol. 152: 5368 (1994).

Alternatively, the bispecific antibody may be a "linear antibody" produced as described in Zapata et al. Protein Eng. 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H -C_H1-V_H -C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are also contemplated. For example, trispecific antibodies can be prepared. (Tutt et al., J. Immunol. 147:60 (1991))

In certain embodiments, the humanized or variant anti-M-CSF antibody is an antibody fragment. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., Journal of Biochemical and Biophysical Methods 24:107-117 (1992) and Brennan et al., Science 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. Better et al., Science 240: 1041-

1043 (1988) disclose secretion of functional antibody fragments from bacteria (*see, e.g.*, Better et al., Skerra et al. Science 240: 1038-1041 (1988)). For example, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). In another embodiment, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, Fv, Fab or $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

An "isolated" antibody is one that has been identified and separated and for recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

For a detailed description of the structure and generation of antibodies, see Roth, D.B., and Craig, N.L., *Cell*, 94:411-414 (1998), and United States Patent No. 6,255,458, herein incorporated by reference in its entirety. Briefly, the process for generating DNA encoding the heavy and light chain immunoglobulin genes occurs primarily in developing B-cells. Prior to the rearranging and joining of various immunoglobulin gene segments, the V, D, J and constant (C) gene segments are found generally in relatively close proximity on a single chromosome. During B-cell-differentiation, one of each of the appropriate family members of the V, D, J (or only V and J in the case of light chain genes) gene segments are recombined to form functionally rearranged heavy and light immunoglobulin genes. This gene segment rearrangement process appears to be sequential. First, heavy chain D-to-J joints are made, followed by heavy chain V-to-DJ joints and light chain V-to-J joints.

The recombination of variable region gene segments to form functional heavy

and light chain variable regions is mediated by recombination signal sequences (RSS's) that flank recombinationally competent V, D and J segments. RSS's necessary and sufficient to direct recombination, comprise a dyad-symmetric heptamer, an AT-rich nonamer and an intervening spacer region of either 12 or 23 base pairs. These signals are conserved among the different loci and species that carry out D-J (or V-J) recombination and are functionally interchangeable. See Oettinger, et al. (1990), Science, 248, 1517-1523 and references cited therein. The heptamer comprises the sequence CACAGTG or its analogue followed by a spacer of unconserved sequence and then a nonamer having the sequence ACAAAAACC or its analogue. These sequences are found on the J, or downstream side, of each V and D gene segment. Immediately preceding the germline D and J segments are again two recombination signal sequences, first the nonamer and then the heptamer again separated by an unconserved sequence. The heptameric and nonameric sequences following a V_L, V_H or D segment are complementary to those preceding the J_L, D or J_H segments with which they recombine. The spacers between the heptameric and nonameric sequences are either 12 base pairs long or between 22 and 24 base pairs long.

In addition to the rearrangement of V, D and J segments, further diversity is generated in the primary repertoire of immunoglobulin heavy and light chain by way of variable recombination at the locations where the V and J segments in the light chain are joined and where the D and J segments of the heavy chain are joined. Such variation in the light chain typically occurs within the last codon of the V gene segment and the first codon of the J segment. Similar imprecision in joining occurs on the heavy chain chromosome between the D and J_H segments and may extend over as many as 10 nucleotides. Furthermore, several nucleotides may be inserted between the D and J_H and between the V_H and D gene segments which are not encoded by genomic DNA. The addition of these nucleotides is known as N-region diversity.

The net effect of such rearrangements in the variable region gene segments and the variable recombination which may occur during such joining is the production of a primary antibody repertoire.

"Fv" is the minimum antibody fragment that contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the VH VI dimer. Collectively, the six CDRs confer antigen-binding specificity to the

antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab fragments differ from Fab' fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them.

By "neutralizing antibody" is meant an antibody molecule that is able to eliminate or significantly reduce an effecter function of a target antigen to which is binds. Accordingly, a "neutralizing" anti-target antibody is capable of eliminating or significantly reducing an effecter function, such as enzyme activity, ligand binding, or intracellular signaling.

As provided herein, the compositions for and methods of treating cancer metastasis and/or bone loss associated with cancer metastasis may utilize one or more antibody used singularly or in combination with other therapeutics to achieve the desired effects. Antibodies according to the present invention may be isolated from an animal producing the antibody as a result of either direct contact with an environmental antigen or immunization with the antigen. Alternatively, antibodies may be produced by recombinant DNA methodology using one of the antibody expression systems well known in the art (See, e.g., Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory (1988)). Such antibodies may include recombinant IgGs, chimeric fusion proteins having immunoglobulin derived sequences or "humanized" antibodies that may all be used for the treatment of cancer metastasis and/or bone loss associated with cancer metastasis according to the present invention. In addition to intact, full-length molecules, the term "antibody" also refers to fragments thereof (such as, e.g., scFv, Fv, Fd, Fab, Fab' and F(ab)'2 fragments) or multimers or aggregates of intact molecules and/or fragments that bind to M-CSF (or M-CSFR). These antibody fragments bind antigen and may be derivatized to exhibit structural features that facilitate clearance and uptake, e.g., by incorporation of galactose residues.

In one embodiment of the present invention, M-CSF monoclonal antibodies

may be prepared essentially as described in Halenbeck et al. U.S. Pat. No. 5,491,065 (1997), incorporated herein by reference. Exemplary M-CSF monoclonal antibodies include those that bind to an apparent conformational epitope associated with recombinant or native dimeric M-CSF with concomitant neutralization of biological activity. These antibodies are substantially unreactive with biologically inactive forms of M-CSF including monomeric and chemically derivatized dimeric M-CSF.

In other embodiments of the present invention, humanized anti-M-CSF monoclonal antibodies are provided. The phrase "humanized antibody" refers to an antibody derived from a non-human antibody, typically a mouse monoclonal antibody. Alternatively, a humanized antibody may be derived from a chimeric antibody that retains or substantially retains the antigen binding properties of the parental, non-human, antibody but which exhibits diminished immunogenicity as compared to the parental antibody when administered to humans. The phrase "chimeric antibody," as used herein, refers to an antibody containing sequence derived from two different antibodies (see, e.g., U.S. Patent No. 4,816,567) which typically originate from different species. Most typically, chimeric antibodies comprise human and murine antibody fragments, generally human constant and mouse variable regions.

The phrase "complementarity determining region" or the term "CDR" refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site (See, e.g., Chothia et al., J. Mol. Biol. 196:901 917 (1987); Kabat et al., U.S. Dept. of Health and Human Services NIH Publication No. 91 3242 (1991)). The phrase "constant region" refers to the portion of the antibody molecule that confers effector functions. In the present invention, mouse constant regions are preferably substituted by human constant regions. The constant regions of the subject humanized antibodies are derived from human immunoglobulins. The heavy chain constant region can be selected from any of the five isotypes: alpha, delta, epsilon, gamma or mu.

The antibodies of the present invention are said to be immunospecific or specifically binding if they bind to antigen with a K_a of greater than or equal to about 10^6M^{-1} preferably greater than or equal to about 10^7M^{-1}, more preferably greater than or equal to about 10^8M^{-1}, and most preferably greater than or equal to about 10^9M^{-1}, $10^{10}M^{-1}$, $10^{11}M^{-1}$ or $10^{12}M^{-1}$. The anti-M-CSF antibodies may bind to different naturally occurring forms of M-CSF, including those expressed by the host's/subject's tissues as well as that expressed by the tumor. The monoclonal antibodies disclosed herein have affinity for M-CSF and are

characterized by a dissociation equilibrium constant (Kd) of at least 10^{-4} M, preferably at least about 10^{-7} M to about 10^{-8} M, more preferably at least about 10^{-8} M, 10^{-10}M, 10^{-11}M or 10^{-12}M. Such affinities may be readily determined using conventional techniques, such as by equilibrium dialysis; by using the BIAcore 2000 instrument, using general procedures outlined by the manufacturer; by radioimmunoassay using ^{125}I labeled M-CSF; or by another method known to the skilled artisan. The affinity data may be analyzed, for example, by the method of Scatchard et al., Ann N.Y. Acad. Sci., 51:660 (1949). Thus, it will be apparent that preferred M-CSF antibodies will exhibit a high degree of specificity for M-CSF and will bind with substantially lower affinity to other molecules. Preferred antibodies bind M-CSF with a similar affinity as murine RX1 of Figure 4 binds to M-CSF, exhibit low immunogenicity, and inhibit metastasis of cancer cells when tested in metastatic disease animal models.

The antigen to be used for production of antibodies may be, e.g., intact M-CSF or a fragment of M-CSF that retains the desired epitope, optionally fused to another polypeptide that allows the epitope to be displayed in its native conformation. Alternatively, cells expressing M-CSF at their cell surface can be used to generate antibodies. Such cells can be transformed to express M-CSF or may be other naturally occurring cells that express M-CSF. Other forms of M-CSF useful for generating antibodies will be apparent to those skilled in the art.

Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. An improved antibody response may be obtained by conjugating the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride or other agents known in the art.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later, the animals are boosted with 1/5 to {fraction

(1/10)} the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. At 7-14 days post-booster injection, the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., Nature, 256:495 (1975), or may be made by recombinant DNA methods.

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as herein described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133: 3001 (1984) ;Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)). Exemplary murine myeloma lines include those derived from MOP-21 and M.C.-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells

available from the American Type Culture Collection, Rockville, Md. USA.

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The binding affinity of the monoclonal antibody can, for example, be determined by Scatchard analysis (Munson et al., Anal. Biochem., 107:220 (1980)).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal. The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

Recombinant Production of Antibodies

DNA encoding the monoclonal antibodies may be isolated and sequenced from the hybridoma cells using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). Sequence determination will generally require isolation of at least a portion of the gene or cDNA of interest. Usually this requires cloning the DNA or, preferably, mRNA (i.e., cDNA) encoding the monoclonal antibodies. Cloning is carried out using standard techniques (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, which is incorporated herein by reference). For example, a cDNA library may be constructed by reverse transcription of polyA+ mRNA, preferably membrane-associated mRNA, and the library screened using probes specific for human immunoglobulin polypeptide gene sequences. In a preferred embodiment, however, the polymerase chain reaction (PCR) is used to amplify cDNAs (or portions of full-lenght cDNAs) encoding an immunoglobulin gene segment of interest (e.g., a

light chain variable segment). The amplified sequences can be readily cloned into any suitable vector, e.g., expression vectors, minigene vectors, or phage display vectors. It will be appreciated that the particular method of cloning used not critical, so long as it is possible to determine the sequence of some portion of the immunoglobulin polypeptide of interest. As used herein, an "isolated" nucleic acid molecule or "isolated" nucleic acid sequence is a nucleic acid molecule that is either (1) identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid or (2) cloned, amplified, tagged, or otherwise distinguished from background nucleic acids such that the sequence of the nucleic acid of interest can be determined, is considered isolated. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells that ordinarily express the antibody where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

One source for RNA used for cloning and sequencing is a hybridoma produced by obtaining a B cell from the transgenic mouse and fusing the B cell to an immortal cell. An advantage of using hybridomas is that they can be easily screened, and a hybridoma that produces a human monoclonal antibody of interest selected. Alternatively, RNA can be isolated from B cells (or whole spleen) of the immunized animal. When sources other than hybridomas are used, it may be desirable to screen for sequences encoding immunoglobulins or immunoglobulin polypeptides with specific binding characteristics. One method for such screening is the use of phage display technology. Phage display is described in e.g., Dower et al., WO 91/17271, McCafferty et al., WO 92/01047, and Caton and Koprowski, Proc. Natl. Acad. Sci. USA, 87:6450-6454 (1990), each of which is incorporated herein by reference. In one embodiment using phage display technology, cDNA from an immunized transgenic mouse (e.g., total spleen cDNA) is isolated, the polymerase chain reaction is used to amplify a cDNA sequences that encode a portion of an immunoglobulin polypeptide, e.g., CDR regions, and the amplified sequences are inserted into a phage vector. cDNAs encoding peptides of interest, e.g., variable region peptides with desired binding characteristics, are identified by standard techniques such as panning.

The sequence of the amplified or cloned nucleic acid is then determined. Typically the sequence encoding an entire variable region of the immunoglobulin polypeptide

is determined, however, it will sometimes by adequate to sequence only a portion of a variable region, for example, the CDR-encoding portion. Typically the portion sequenced will be at least 30 bases in length, more often based coding for at least about one-third or at least about one-half of the length of the variable region will be sequenced.

Sequencing can be carried out on clones isolated from a cDNA library, or, when PCR is used, after subcloning the amplified sequence or by direct PCR sequencing of the amplified segment. Sequencing is carried out using standard techniques (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Guide, Vols 1-3, Cold Spring Harbor Press, and Sanger, F. et al. (1977) Proc. Natl. Acad. Sci. USA 74: 5463-5467, which is incorporated herein by reference). By comparing the sequence of the cloned nucleic acid with published sequences of human immunoglobulin genes and cDNAs, one of skill will readily be able to determine, depending on the region sequenced, (i) the germline segment usage of the hybridoma immunoglobulin polypeptide (including the isotype of the heavy chain) and (ii) the sequence of the heavy and light chain variable regions, including sequences resulting from N-region addition and the process of somatic mutation. One source of immunoglobulin gene sequence information is the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md.

Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies is well known in the art.

Expression control sequences refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably

linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

Cell, cell line, and cell culture are often used interchangeably and all such designations herein include progeny. Transformants and transformed cells include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

In an alternative embodiment, the amino acid sequence of an immunoglobulin of interest may be determined by direct protein sequencing. Suitable encoding nucleotide sequences can be designed according to a universal codon table.

Amino acid sequence variants of the desired antibody may be prepared by introducing appropriate nucleotide changes into the encoding DNA, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antibodies. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the humanized or variant antibody, such as changing the number or position of glycosylation sites.

Nucleic acid molecules encoding amino acid sequence variants of the antibody are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antibody.

The invention also provides isolated nucleic acid encoding antibodies of the invention, optionally operably linked to control sequences recognized by a host cell, vectors

and host cells comprising the nucleic acids, and recombinant techniques for the production of the antibodies, which may comprise culturing the host cell so that the nucleic acid is expressed and, optionally, recovering the antibody from the host cell culture or culture medium.

For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the monoclonal antibody is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more selective marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(1) Signal sequence component

The antibody of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. If prokaryotic host cells do not recognize and process the native antibody signal sequence, the signal sequence may be substituted by a signal sequence selected, for example, from the group of the pectate lyase (e.g., pelB) alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including Saccharomyces and Kluyveromyces α-factor leaders), or acid phosphatase leader, the C. albicans glucoamylase leader, or the signal described in WO90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the antibody.

(2) Origin of replication component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning

vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is suitable for yeast, and various viral origins are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

(3) Selective marker component

Expression and cloning vectors may contain a selective gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for Bacilli.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs methotrexate, neomycin, histidinol, puromycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the antibody-encoding nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity.

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding antibody of the invention, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, *e.g.*, kanamycin,

neomycin, or G418. *See* U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature, 282: 39 (1979)). The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, Genetics, 85: 12 (1977). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene. *Ura*3-deficient yeast strains are complemented by plasmids bearing the *ura*3 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of Kluyveromyces yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for K. lactis. Van den Berg, Bio/Technology, 8: 135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of Kluyveromyces have also been disclosed. Fleer et al, Bio/Technology, 9: 968-975 (1991).

(4) Promoter component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the antibody-encoding nucleic acid. Promoters suitable for use with prokaryotic hosts include the arabinose (*e.g.*, araB) promoter phoA promoter , β-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and hybrid promoters such as the tac promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the antibody of the invention.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the

promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Antibody transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, most preferably cytomegalovirus, a retrovirus, hepatitis-B virus, Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. *See* also Reyes et al., *Nature 297*: 598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the rous sarcoma virus long terminal repeat can be used as the promoter.

(5) Enhancer element component

Transcription of a DNA encoding the antibody of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, alpha-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell

virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. *See* also Yaniv, *Nature 297*: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the antibody-encoding sequence, but is preferably located at a site 5' from the promoter.

(6) Transcription termination component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding antibody. One useful transcription termination component is the bovine growth hormone polyadenylation region. *See* WO94/11026 and the expression vector disclosed therein. Another is the mouse immunoglobulin light chain transcription terminator.

(7) Selection and transformation of host cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *Enterobacteriaceae* such as *Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g., B. licheniformis* 41 P disclosed in DD 266,710 published Apr. 12, 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such

as, *e.g.*, *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; *yarrowia* (EP 402,226); *Pichia pastors* (EP 183,070); *Candida*; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, *e.g.*, *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated antibody are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera frugiperda (caterpillar), Aedes aegypti (mosquito), Aedes albopictus (mosquito), Drosophila melanogaster (fruitfly), and Bombyx mori have been identified. A variety of viral strains for transfection are publicly available, *e.g.*, the L-1 variant of Autographa californica NPV and the Bm-5 strain of Bombyx mori NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of Spodoptera frugiperda cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become routine procedure. Examples of useful mammalian host cell lines are Chinese hamster ovary cells, including CHOK1 cells (ATCC CCL61), DXB-11, DG-44, and Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., Proc. Natl. Acad. Sci. USA 77: 4216 (1980)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, [Graham et al., *J. Gen Virol. 36*: 59 (1977)]; baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23: 243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y Acad. Sci. 383: 44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed or transfected with the above-described expression or cloning vectors for antibody production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. In addition, novel vectors and transfected cell lines with multiple copies of transcription units separated by a selective marker are particularly useful and preferred for the expression of antibodies that target M-CSF.

(8) Culturing the host cells

The host cells used to produce the antibody of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., Meth. Enz. 58: 44 (1979), Barnes et al., Anal. Biochem. 102: 255 (1980), U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO90103430; WO 87/00195; or U.S. Patent Re. No. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(9) Purification of antibody

When using recombinant techniques, the antibody can be produced intracellularly, in the periplasmic space, or directly secreted into the medium, including from microbial cultures. If the antibody is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Better et al. Science 240: 1041-1043 (1988); ICSU Short Reports 10: 105 (1990); and Proc. Natl. Acad. Sci. USA 90: 457-461 (1993) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. (*See* also, [Carter et al.,

Bio/Technology 10: 163-167 (1992)].

The antibody composition prepared from microbial or mammalian cells can be purified using, for example, hydroxylapatite chromatography cation or avian exchange chromatography, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human γ1, γ2, or γ4 heavy chains (Lindmark et al., *J. Immunol. Meth. 62*: 1-13 (1983)). Protein G is recommended for all mouse isotypes and for human γ3 (Guss et al., EMBO J. 5: 15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. Where the antibody comprises a C_H 3 domain, the Bakerbond ABX™resin (J. T. Baker, Phillipsburg, N.J.) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Chimeric and humanized antibodies

Because chimeric or humanized antibodies are less immunogenic in humans than the parental mouse monoclonal antibodies, they can be used for the treatment of humans with far less risk of anaphylaxis. Thus, these antibodies may be preferred in therapeutic applications that involve in vivo administration to a human.

Chimeric monoclonal antibodies, in which the variable Ig domains of a mouse monoclonal antibody are fused to human constant Ig domains, can be generated using standard procedures known in the art (See Morrison, S. L., et al. (1984) Chimeric Human Antibody Molecules; Mouse Antigen Binding Domains with Human Constant Region Domains, Proc. Natl. Acad. Sci. USA 81, 6841-6855; and, Boulianne, G. L., et al, Nature 312, 643-646 . (1984)). Although some chimeric monoclonal antibodies have proved less immunogenic in humans, the mouse variable Ig domains can still lead to a significant human anti-mouse response.

Humanized antibodies may be achieved by a variety of methods including, for example: (1) grafting the non-human complementarity determining regions (CDRs) onto a human framework and constant region (a process referred to in the art as humanizing through "CDR grafting"), or, alternatively, (2) transplanting the entire non-human variable domains, but "cloaking" them with a human-like surface by replacement of surface residues (a process referred to in the art as "veneering"). In the present invention, humanized antibodies will include both "humanized" and "veneered" antibodies. These methods are disclosed in, e.g., Jones et al., Nature 321:522 525 (1986); Morrison et al., Proc. Natl. Acad. Sci., U.S.A., 81:6851 6855 (1984); Morrison and Oi, Adv. Immunol., 44:65 92 (1988); Verhoeyer et al., Science 239:1534 1536 (1988); Padlan, Molec. Immun. 28:489 498 (1991); Padlan, Molec. Immunol. 31(3):169 217 (1994); and Kettleborough, C.A. et al., Protein Eng. 4(7):773 83 (1991) each of which is incorporated herein by reference.

In particular, a rodent antibody on repeated in vivo administration in man either alone or as a conjugate will bring about an immune response in the recipient against the rodent antibody; the so-called HAMA response (Human Anti Mouse Antibody). The HAMA response may limit the effectiveness of the pharmaceutical if repeated dosing is required. The immunogenicity of the antibody may be reduced by chemical modification of the antibody with a hydrophilic polymer such as polyethylene glycol or by using the methods of genetic engineering to make the antibody binding structure more human like. For example, the gene sequences for the variable domains of the rodent antibody which bind CEA can be substituted for the variable domains of a human myeloma protein, thus producing a recombinant chimaeric antibody. These procedures are detailed in EP 194276, EP 0120694, EP 0125023, EP 0171496, EP 0173494 and WO 86/01533. Alternatively the gene sequences of the CDRs of the rodent antibody may be isolated or synthesized and substituted for the corresponding sequence regions of a homologous human antibody gene, producing a human antibody with the specificity of the original rodent antibody. These procedures are described in EP 023940, WO 90/07861 and WO91/09967. Alternatively a large number of the surface residues of the variable domain of the rodent antibody may be changed to those residues normally found on a homologous human antibody, producing a rodent antibody which has a surface `veneer` of residues and which will therefore be recognized as self by the human body. This approach has been demonstrated by Padlan et.al. (1991) Mol. Immunol. 28, 489.

CDR grafting involves introducing one or more of the six CDRs from the mouse heavy and light chain variable Ig domains into the appropriate four framework regions

of human variable Ig domains is also called CDR grafting. This technique (Riechmann, L., et al., Nature 332, 323 (1988)), utilizes the conserved framework regions (FR1-FR4) as a scaffold to support the CDR loops which are the primary contacts with antigen. A disadvantage of CDR grafting, however, is that it can result in a humanized antibody that has a substantially lower binding affinity than the original mouse antibody, because amino acids of the framework regions can contribute to antigen binding, and because amino acids of the CDR loops can influence the association of the two variable Ig domains. To maintain the affinity of the humanized monoclonal antibody, the CDR grafting technique can be improved by choosing human framework regions that most closely resemble the framework regions of the original mouse antibody, and by site-directed mutagenesis of single amino acids within the framework or CDRs aided by computer modeling of the antigen binding site (e.g., Co, M. S., et al. (1994), J. Immunol. 152, 2968-2976).

One method of humanizing antibodies comprises aligning the non-human heavy and light chain sequences to human heavy and light chain sequences, selecting and replacing the non-human framework with a human framework based on such alignment, molecular modeling to predict the conformation of the humanized sequence and comparing to the conformation of the parent antibody. This process is followed by repeated back mutation of residues in the CDR region which disturb the structure of the CDRs until the predicted conformation of the humanized sequence model closely approximates the conformation of the non-human CDRs of the parent non-human antibody.. Such humanized antibodies may be further derivatized to facilitate uptake and clearance, e.g., via Ashwell receptors (See, e.g., U.S. Patent Nos. 5,530,101 and 5,585,089 which patents are incorporated herein by reference).

A number of humanizations of mouse monoclonal antibodies by rational design have been reported (See, for example, 20020091240 published July 11, 2002, WO 92/11018 and U.S. Patent No., 5,693,762, U.S. Patent No. 5,766,866.

Amino acid sequence variants

A useful method for identification of certain residues or regions of the antibody that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells Science, 244:1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably

alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed antibody variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intra-sequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antibody with an N-terminal methionyl residue or the antibody (including antibody fragment) fused to an epitope tag or a salvage receptor epitope. Other insertional variants of the antibody molecule include the fusion to a polypeptide which increases the serum half-life of the antibody, e.g. at the N-terminus or C-terminus.

The term "epitope tagged" refers to the antibody fused to an epitope tag. The epitope tag polypeptide has enough residues to provide an epitope against which an antibody there against can be made, yet is short enough such that it does not interfere with activity of the antibody. The epitope tag preferably is sufficiently unique so that the antibody there against does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., *Mol. Cell. Biol. 8*: 2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., *Mol. Cell. Biol. 5(12)*: 3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., *Protein Engineering 3(6)*: 547-553 (1990)]. Other exemplary tags are a poly-histidine sequence, generally around six histidine residues, that permits isolation of a compound so labeled using nickel chelation. Other labels and tags, such as the FLAG® tag (Eastman Kodak, Rochester, NY), well known and routinely used in the art, are embraced by the invention.

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the in vivo serum half-life of the IgG molecule.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antibody molecule removed and a different residue inserted in its place. Substitutional mutagenesis within any of the hypervariable or CDR regions or framework regions is contemplated. Conservative substitutions are shown in Table 1. The most conservative substitution is found under the heading of "preferred substitutions". If such substitutions result in no change in biological activity, then more substantial changes, denominated "exemplary substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

TABLE 1

Original	Exemplary	Preferred Residue Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; asp, lys; gln	arg
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q)	asn; glu	asn
Glu (E)	asp; gln	asp
Gly (G)	ala	
His (H)	asn; gln; lys; arg	
Ile (I)	leu; val; met; ala;	leu
	phe;	norleucine
Leu (L)	norleucine; ile; val;	ile
	met; ala; phe	
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	
Pro (P)	ala	

Ser (S)	thr	
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antibody are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Conservative substitutions involve replacing an amino acid with another member of its class. Non-conservative substitutions involve replacing a member of one of these classes with a member of another class.

Any cysteine residue not involved in maintaining the proper conformation of the humanized or variant antibody also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) may be added to the antibody to improve its stability (particularly where the antibody is an antibody fragment such as an Fv fragment).

Affinity maturation involves preparing and screening antibody variants that have substitutions within the CDRs of a parent antibody and selecting variants that have improved biological properties such as binding affinity relative to the parent antibody. A

convenient way for generating such substitutional variants is affinity maturation using phage display. Briefly, several hypervariable region sites (e.g. 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (e.g. binding affinity).

Alanine scanning mutagenesis can be performed to identify hypervariable region residues that contribute significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Antibody variants can also be produced that have a modified glycosylation pattern relative to the parent antibody, for example, deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. The presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. Thus, N-linked glycosylation sites may be added to an antibody by altering the amino acid sequence such that it contains one or more of these tripeptide sequences. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used. O-linked glycosylation sites may be added to an antibody by inserting or substituting one or more serine or threonine residues to the sequence of the original antibody. By way of example, the amino acids of RX1 at positions 41-43 of Figure 4A (NGS) may be retained. alternatively, only amino acids 41 and 42 (NG) may be retained.

Ordinarily, amino acid sequence variants of the human engineered antibody will have an amino acid sequence having at least 60% amino acid sequence identity with the original human engineered antibody amino acid sequences of either the heavy or the light chain (*e.g.*, as in any of Figures 19B through 22B) more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%, including for example, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and 100%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the human engineered residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions (as defined in Table I above) as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the antibody sequence shall be construed as affecting sequence identity or homology. Thus, sequence identity can be determined by standard methods that are commonly used to compare the similarity in position of the amino acids of two polypeptides. Using a computer program such as BLAST or FASTA, two polypeptides are aligned for optimal matching of their respective amino acids (either along the full length of one or both sequences, or along a pre-determined portion of one or both sequences). The programs provide a default opening penalty and a default gap penalty, and a scoring matrix such as PAM 250 [a standard scoring matrix; see Dayhoff et al., in *Atlas of Protein Sequence and Structure*, vol. 5, supp. 3 (1978)] can be used in conjunction with the computer program. For example, the percent identity can then be calculated as: the total number of identical matches multiplied by 100 and then divided by the sum of the length of the longer sequence within the matched span and the number of gaps introduced into the longer sequences in order to align the two sequences.

Other modifications of the antibody are contemplated. For example, it may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating cancer, for example. For example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). *See* Caron et al., J. Exp Med. 176: 1191-1195 (1992) and Shopes, B. J. Immunol. 148: 2918-2922 (1992). Homodimeric antibodies with

enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff et al., Cancer Research 53: 2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. *See* Stevenson et al., *Anti-Cancer Drug Design 3*: 219-230 (1989). In addition, it has been shown that sequences within the CDR can cause an antibody to bind to MHC Class II and trigger an unwanted helper T-cell response. A conservative substitution can allow the antibody to retain binding activity yet lose its ability to trigger an unwanted T-cell response.

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the antibody fragment in order to increase its serum half-life, for example, adding molecules such as PEG or other water soluble polymers, including polysaccharide polymers, to antibody fragments to increase the half-life. This may also be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.*, by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at either end or in the middle, *e.g.*, by DNA or peptide synthesis) (*see, e.g.*, WO96/32478).

The salvage receptor binding epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc region (e.g., of an IgG) and transferred to the CH1, CH3, or VH region, or more than one such region, of the antibody. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C.sub.L region or V.sub.L region, or both, of the antibody fragment. See also International applications WO 97/34631 and WO 96/32478 which describe Fc variants and their interaction with the salvage receptor.

Thus, antibodies of the invention may comprise a human or humanized Fc portion, a human or humanized consensus Fc portion, or a variant thereof that retains the ability to interact with the Fc salvage receptor, including variants in which cysteines involved in disulfide bonding are modified or removed, and/or in which the a met is added at the N-terminus and/or one or more of the N-terminal 20 amino acids are removed, and/or regions that interact with complement, such as the C1q binding site, are removed, and/or the ADCC

site is removed [see, e.g., Molec. Immunol. 29 (5): 633-9 (1992)].

Human and humanized antibodies

Humanized or human antibodies to M-CSF can also be produced using transgenic animals that have no endogenous immunoglobulin production and are engineered to contain human immunoglobulin loci. For example, WO 98/24893 discloses transgenic animals having a human Ig locus wherein the animals do not produce functional endogenous immunoglobulins due to the inactivation of endogenous heavy and light chain loci. WO 91/741 also discloses transgenic non-primate mammalian hosts capable of mounting an immune response to an immunogen, wherein the antibodies have primate constant and/or variable regions, and wherein the endogenous immunoglobulin encoding loci are substituted or inactivated. WO 96/30498 discloses the use of the Cre/Lox system to modify the immunoglobulin locus in a mammal, such as to replace all or a portion of the constant or variable region to form a modified antibody molecule. WO 94/02602 discloses non-human mammalian hosts having inactivated endogenous Ig loci and functional human Ig loci. U.S. Patent No. 5,939,598 discloses methods of making transgenic mice in which the mice lack endogenous heavy chains, and express an exogenous immunoglobulin locus comprising one or more xenogeneic constant regions.

Using a transgenic animal described above, an immune response can be produced to a selected antigenic molecule, and antibody producing cells can be removed from the animal and used to produce hybridomas that secrete human monoclonal antibodies. Immunization protocols, adjuvants, and the like are known in the art, and are used in immunization of, for example, a transgenic mouse as described in WO 96/33735. This publication discloses monoclonal antibodies against a variety of antigenic molecules including IL 6, IL 8, TNFa, human CD4, L selectin, gp39, and tetanus toxin. The monoclonal antibodies can be tested for the ability to inhibit or neutralize the biological activity or physiological effect of the corresponding protein. WO 96/33735 discloses that monoclonal antibodies against IL-8, derived from immune cells of transgenic mice immunized with IL-8, blocked IL-8 induced functions of neutrophils. Human monoclonal antibodies with specificity for the antigen used to immunize transgenic animals are also disclosed in WO 96/34096 and U.S. patent application no. 20030194404; and U.S. patent application no. 20030031667).

See also Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993); and U.S. Pat. No. 5,591,669, U.S. Patent No. 5,589,369, U.S. Patent No. 5,545,807; and U.S Patent Application No. 20020199213. U.S. Patent Application No. and 20030092125 describes methods for biasing the immune response of an animal to the desired epitope. Human antibodies may also be generated by *in vitro* activated B cells (see U.S. Pat. Nos. 5,567,610 and 5,229,275).

The development of technologies for making repertoires of recombinant human antibody genes, and the display of the encoded antibody fragments on the surface of filamentous bacteriophage, has provided a means for making human antibodies directly. The antibodies produced by phage technology are produced as antigen binding fragments-usually Fv or Fab fragments-in bacteria and thus lack effector functions. Effector functions can be introduced by one of two strategies: The fragments can be engineered either into complete antibodies for expression in mammalian cells, or into bispecific antibody fragments with a second binding site capable of triggering an effector function.

Typically, the Fd fragment (V_H-C_H1) and light chain (V_L-C_L) of antibodies are separately cloned by PCR and recombined randomly in combinatorial phage display libraries, which can then be selected for binding to a particular antigen. The Fab fragments are expressed on the phage surface, i.e., physically linked to the genes that encode them. Thus, selection of Fab by antigen binding co-selects for the Fab encoding sequences, which can be amplified subsequently. By several rounds of antigen binding and re-amplification, a procedure termed panning, Fab specific for the antigen are enriched and finally isolated.

In 1994, an approach for the humanization of antibodies, called "guided selection", was described. Guided selection utilizes the power of the phage display technique for the humanization of mouse monoclonal antibody (See Jespers, L. S., et al., Bio/Technology 12, 899-903 (1994)). For this, the Fd fragment of the mouse monoclonal antibody can be displayed in combination with a human light chain library, and the resulting hybrid Fab library may then be selected with antigen. The mouse Fd fragment thereby provides a template to guide the selection. Subsequently, the selected human light chains are combined with a human Fd fragment library. Selection of the resulting library yields entirely human Fab.

A variety of procedures have been described for deriving human antibodies

from phage-display libraries (See, for example, Hoogenboom et al., J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol, 222:581-597 (1991); U.S. Pat. Nos. 5,565,332 and 5,573,905; Clackson, T., and Wells, J. A., TIBTECH 12, 173-184 (1994)). In particular, in vitro selection and evolution of antibodies derived from phage display libraries has become a powerful tool (See Burton, D. R., and Barbas III, C. F., Adv. Immunol. 57, 191-280 (1994); and, Winter, G., et al., Annu. Rev. Immunol. 12, 433-455 (1994); U.S. patent application no. 20020004215 and WO92/01047; U.S. patent application no. 20030190317 published October 9, 2003 and U.S. Patent No. 6,054,287; U.S. Patent No. 5,877,293.

Watkins, "Screening of Phage-Expressed Antibody Libraries by Capture Lift," Methods in Molecular Biology, Antibody Phage Display: Methods and Protocols 178: 187-193, and U.S. patent application no. 200120030044772 published March 6, 2003 describe methods for screening phage-expressed antibody libraries or other binding molecules by capture lift, a method involving immobilization of the candidate binding molecules on a solid support.

The antibody products may be screened for activity and for suitability in the treatment methods of the invention using assays as described in the section entitled "Screening Methods" herein or using any suitable assays known in the art.

Other covalent modifications

Covalent modifications of the antibody are also included within the scope of this invention. They may be made by chemical synthesis or by enzymatic or chemical cleavage of the antibody, if applicable. Other types of covalent modifications of the antibody are introduced into the molecule by reacting targeted amino acid residues of the antibody with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, .alpha.-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH

5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino-terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing .alpha.-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N.dbd.C.dbd.N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the .alpha.-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, Proteins:

Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification involves chemically or enzymatically coupling glycosides to the antibody. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO87/05330 published 11 Sep. 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of any carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact. Chemical deglycosylation is described by Hakimuddin, et al. Arch. Biochem. Biophys. 259: 52 (1987) and by Edge et al. Anal. Biochem., 118: 131 (1981). Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. Meth. Enzymol. 138: 350 (1987).

Another type of covalent modification of the antibody comprises linking the antibody to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, polyoxyethylated polyols, polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol, polyoxyalkylenes, or polysaccharide polymers such as dextran. Such methods are known in the art, see, e.g. U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192, 4,179,337, 4,766,106, 4,179,337, 4,495,285, 4,609,546or EP 315 456.

Gene Therapy

Delivery of a therapeutic antibody to appropriate cells can be effected via gene therapy ex vivo, in situ, or in vivo by use of any suitable approach known in the art, including by use of physical DNA transfer methods (e.g., liposomes or chemical treatments) or by use of viral vectors (e.g., adenovirus, adeno-associated virus, or a retrovirus). For example, for in

vivo therapy, a nucleic acid encoding the desired antibody, either alone or in conjunction with a vector, liposome, or precipitate may be injected directly into the subject, and in some embodiments, may be injected at the site where the expression of the antibody compound is desired. For ex vivo treatment, the subject's cells are removed, the nucleic acid is introduced into these cells, and the modified cells are returned to the subject either directly or, for example, encapsulated within porous membranes which are implanted into the patient. See, e.g. U.S. Pat. Nos. 4,892,538 and 5,283,187. There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or in vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, and calcium phosphate precipitation. A commonly used vector for ex vivo delivery of a nucleic acid is a retrovirus.

Other in vivo nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno-associated virus) and lipid-based systems. The nucleic acid and transfection agent are optionally associated with a microparticle. Exemplary transfection agents include calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, quaternary ammonium amphiphile DOTMA ((dioleoyloxypropyl) trimethylammonium bromide, commercialized as Lipofectin by GIBCO-BRL))(Felgner et al, (1987) Proc. Natl. Acad. Sci. USA 84, 7413-7417; Malone et al. (1989) Proc. Natl Acad. Sci. USA 86 6077-6081); lipophilic glutamate diesters with pendent trimethylammonium heads (Ito et al. (1990) Biochem. Biophys. Acta 1023, 124-132); the metabolizable parent lipids such as the cationic lipid dioctadecylamido glycylspermine (DOGS, Transfectam, Promega) and dipalmitoylphosphatidyl ethanolamylspermine (DPPES)(J. P. Behr (1986) Tetrahedron Lett. 27, 5861-5864; J. P. Behr et al. (1989) Proc. Natl. Acad. Sci. USA 86, 6982-6986); metabolizable quaternary ammonium salts (DOTB, N-(1-[2,3-dioleoyloxy]propyl)-N,N,N-trimethylammonium methylsulfate (DOTAP)(Boehringer Mannheim), polyethyleneimine (PEI), dioleoyl esters, ChoTB, ChoSC, DOSC)(Leventis et al. (1990) Biochim. Inter. 22, 235-241); 3beta[N-(N', N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol), dioleoylphosphatidyl ethanolamine (DOPE)/3beta[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterolDC-Chol in one to one mixtures (Gao et al., (1991) Biochim. Biophys. Acta 1065, 8-14), spermine, spermidine, lipopolyamines (Behr et al., Bioconjugate Chem, 1994, 5: 382-389), lipophilic polylysines

(LPLL) (Zhou et al., (1991) Biochim. Biophys. Acta 939, 8-18), [[(1,1,3,3-tetramethylbutyl)cre- soxy]ethoxy]ethyl]dimethylbe nzylammonium hydroxide (DEBDA hydroxide) with excess phosphatidylcholine/cholesterol (Ballas et al., (1988) Biochim. Biophys. Acta 939, 8-18), cetyltrimethylammonium bromide (CTAB)/DOPE mixtures (Pinnaduwage et al, (1989) Biochim. Biophys. Acta 985, 33-37), lipophilic diester of glutamic acid (TMAG) with DOPE, CTAB, DEBDA, didodecylammonium bromide (DDAB), and stearylamine in admixture with phosphatidylethanolamine (Rose et al., (1991) Biotechnique 10, 520-525), DDAB/DOPE (TransfectACE, GIBCO BRL), and oligogalactose bearing lipids. Exemplary transfection enhancer agents that increase the efficiency of transfer include, for example, DEAE-dextran, polybrene, lysosome-disruptive peptide (Ohmori N I et al, Biochem Biophys Res Commun Jun. 27, 1997;235(3):726-9), chondroitan-based proteoglycans, sulfated proteoglycans, polyethylenimine, polylysine (Pollard H et al. J Biol Chem, 1998 273 (13):7507-11), integrin-binding peptide CYGGRGDTP, linear dextran nonasaccharide, glycerol, cholesteryl groups tethered at the 3'-terminal internucleoside link of an oligonucleotide (Letsinger, R. L. 1989 Proc Natl Acad Sci USA 86: (17):6553-6), lysophosphatide, lysophosphatidylcholine, lysophosphatidylethanolamine, and 1-oleoyl lysophosphatidylcholine.

In some situations it may be desirable to deliver the nucleic acid with an agent that directs the nucleic acid-containing vector to target cells. Such "targeting" molecules include antibodies specific for a cell-surface membrane protein on the target cell, or a ligand for a receptor on the target cell. Where liposomes are employed, proteins which bind to a cell-surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake. Examples of such proteins include capsid proteins and fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. In other embodiments, receptor-mediated endocytosis can be used. Such methods are described, for example, in Wu et al., 1987 or Wagner et al., 1990. For review of the currently known gene marking and gene therapy protocols, see Anderson 1992. See also WO 93/25673 and the references cited therein. For additional reviews of gene therapy technology, see Friedmann, Science, 244: 1275-1281 (1989); Anderson, Nature, supplement to vol. 392, no 6679, pp. 25-30 (1998); Verma, Scientific American: 68-84 (1990); and Miller, Nature, 357: 455460 (1992).

Screening Methods

Effective therapeutics depend on identifying efficacious agents devoid of significant toxicity. Antibodies may be screened for binding affinity by methods known in the art. For example, gel-shift assays, Western blots, radiolabeled competition assay, co-fractionation by chromatography, co-precipitation, cross linking, ELISA, and the like may be used, which are described in, for example, Current Protocols in Molecular Biology (1999) John Wiley & Sons, NY, which is incorporated herein by reference in its entirety.

To initially screen for antibodies which bind to the desired epitope on M-CSF (*e.g.*, those which block binding of RX1 to M-CSF), a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Routine competitive binding assays may also be used, in which the unknown antibody is characterized by its ability to inhibit binding of M-CSF to an M-CSF specific antibody of the invention. Intact M-CSF, fragments thereof, or linear epitopes such as represented by amino acids 98-105 of M-CSF of Figure 12 can be used. Epitope mapping is described in Champe et al., J. Biol. Chem. 270: 1388-1394 (1995).

It is further contemplated that the antibodies are next tested for their effect on osteoclastogenesis, followed by administration to animals. Compounds potentially useful in preventing or treating bone loss associated with cancer metastasis may be screened using various assays. For instance, a candidate antagonist may first be characterized in a cultured cell system to determine its ability to neutralize M-CSF in inducing osteoclastogenesis. Such a system may include the co-culture of mouse calvarial osteoblasts and spleen cells (Suda et al., Modulation of osteoclast differentiation. Endocr. Rev. 13: 66 80, 1992; Martin and Udagawa, Trends Endocrinol. Metab. 9: 6-12, 1998), the co-culture of mouse stromal cell lines (e.g., MC3T3-G2/PA6 and ST2) and mouse spleen cells (Udagawa et al., Endocrinology 125: 1805 13, 1989), and the co-culture of ST2 cells and bone marrow cells, peripheral blood mononuclear cells or alveolar macrophages (Udagawa et al., Proc. Natl. Acad. Sci. USA 87: 7260 4, 1990; Sasaki et al., Cancer Res. 58: 462 7, 1998; Mancino et al., J. Surg. Res.100: 18-24, 2001). In the absence of any M-CSF antagonist, multinucleated cells formed in such co-cultures satisfy the major criteria of osteoclasts such as tartrate resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) activity, calcitonin receptors, p60C-STC, vitronectin receptors, and the ability to form resorption pits on bone and dentine slices. The presence of an effective M-CSF antagonist inhibits the formation of such multinucleated cells.

In addition to the above co-culture systems, the ability of a candidate M-CSF antibody in inhibiting osteoclastogenesis may be assayed in a stromal cell-free or osteoblast-

free system. The M-CSF required for osteoclastogenesis may be provided by co-cultured metastatic cancer cells (e.g., MDA 231) or conditioned medium from these cancer cells (Mancino et al., J. Surg. Res. 0: 18-24, 2001) or by addition of purified M-CSF.

Efficacy of a given M-CSF antibody in preventing or treating bone loss associated with cancer metastasis may also be tested in any of the animal bone metastasis model systems familiar to those skilled in the art. Such model systems include those involving direct injection of tumor cells into the medullary cavity of bones (Ingall, Proc. Soc. Exp. Biol. Med., 117: 819-22, 1964; Falasko, Clin. Orthop. 169: 20 7, 1982), into the rat abdominal aorta (Powles et al., Br. J. Cancer 28: 316 21, 1973), into the mouse lateral tail vein or into the mouse left ventricle (Auguello et al., Cancer Res. 48: 6876 81, 1988). In the absence of an effective M-CSF antagonist, osteolytic bone metastases formed from injected tumor cells may be determined by radiographs (areas of osteolytic bone lesions) or histochemistry (bone and soft tissues). Sasaki et al., Cancer Res. 55: 3551 7, 1995; Yoneda et al., J. Clin. Invest. 99: 2509 17, 1997. Clohisy and Ramnaraine, Orthop Res. 16: 660 6, 1998. Yin et al., J. Clin. Invest. 103: 197 206, 1999. In the presence of an effective M-CSF antibody, osteolytic bone metastases may be prevented, or inhibited to result in fewer and/or smaller metastases.

The M-CSF antibodies of the present invention may also be useful in preventing or treating cancer metastasis. The effectiveness of a candidate M-CSF antibody in preventing or treating cancer metastasis may be screened using a human amnionic basement membrane invasion model as described in Filderman et al., Cancer Res 52: 36616, 1992. In addition, any of the animal model systems for metastasis of various types of cancers may also be used. Such model systems include, but are not limited to, those described in Wenger et al., Clin. Exp. Metastasis 19: 169 73, 2002; Yi et al., Cancer Res. 62: 917 23, 2002; Tsutsumi et al., Cancer Lett 169: 77-85, 2001; Tsingotjidou et al., Anticancer Res. 21: 971 8, 2001; Wakabayashi et al., Oncology 59: 75 80, 2000; Culp and Kogerman, Front Biosci. 3:D672 83, 1998; Runge et al., Invest Radiol. 32: 212 7; Shioda et al., J. Surg. Oncol. 64: 122 6, 1997; Ma et al., Invest Ophthalmol Vis Sci. 37: 2293 301, 1996; Kuruppu et al., J Gastroenterol Hepatol. 11: 26 32, 1996. In the presence of an effective M-CSF antibody, cancer metastases may be prevented, or inhibited to result in fewer and/or smaller metastases.

The anti-tumor activity of a particular M-CSF antibody, or combination of M-CSF antibodies, may be evaluated *in vivo* using a suitable animal model. For example, xenogenic lymphoma cancer models wherein human lymphoma cells are introduced into

immune compromised animals, such as nude or SCID mice. Efficacy may be predicted using assays which measure inhibition of tumor formation, tumor regression or metastasis, and the like.

In one variation of an *in vitro* assay, the invention provides a method comprising the steps of (a) contacting an immobilized M-CSF with a candidate antibody and (b) detecting binding of the candidate antibody to the M-CSF. In an alternative embodiment, the candidate antibody is immobilized and binding of M-CSF is detected. Immobilization is accomplished using any of the methods well known in the art, including covalent bonding to a support, a bead, or a chromatographic resin, as well as non-covalent, high affinity interaction such as antibody binding, or use of streptavidin/biotin binding wherein the immobilized compound includes a biotin moiety. Detection of binding can be accomplished (i) using a radioactive label on the compound that is not immobilized, (ii) using a fluorescent label on the non-immobilized compound, (iii) using an antibody immunospecific for the non-immobilized compound, (iv) using a label on the non-immobilized compound that excites a fluorescent support to which the immobilized compound is attached, as well as other techniques well known and routinely practiced in the art.

Antibodies that modulate (i.e., increase, decrease, or block) the activity or expression of M-CSF may be identified by incubating a putative modulator with a cell expressing a M-CSF and determining the effect of the putative modulator on the activity or expression of the M-CSF. The selectivity of an antibody that modulates the activity of a M-CSF polypeptide or polynucleotide can be evaluated by comparing its effects on the M-CSF polypeptide or polynucleotide to its effect on other related compounds. Selective modulators may include, for example, antibodies and other proteins, peptides, or organic molecules which specifically bind to M-CSF polypeptides or to a nucleic acid encoding a M-CSF polypeptide. Modulators of M-CSF activity will be therapeutically useful in treatment of diseases and physiological conditions in which normal or aberrant activity of M-CSF polypeptide is involved.

The invention also comprehends high throughput screening (HTS) assays to identify antibodies that interact with or inhibit biological activity (i.e., inhibit enzymatic activity, binding activity, etc.) of a M-CSF polypeptide. HTS assays permit screening of large numbers of compounds in an efficient manner. Cell-based HTS systems are contemplated to investigate the interaction between M-CSF polypeptides and their binding partners. HTS assays are designed to identify "hits" or "lead compounds" having the desired

property, from which modifications can be designed to improve the desired property. Chemical modification of the "hit" or "lead compound" is often based on an identifiable structure/activity relationship between the "hit" and M-CSF polypeptides.

Another aspect of the present invention is directed to methods of identifying antibodies which modulate (i.e., decrease) activity of a M-CSF comprising contacting a M-CSF with an antibody, and determining whether the antibody modifies activity of the M-CSF. The activity in the presence of the test antibody is compared to the activity in the absence of the test antibody. Where the activity of the sample containing the test antibody is lower than the activity in the sample lacking the test antibody, the antibody will have inhibited activity.

A variety of heterologous systems is available for functional expression of recombinant polypeptides that are well known to those skilled in the art. Such systems include bacteria (Strosberg, et al., Trends in Pharmacological Sciences (1992) 13:95-98), yeast (Pausch, Trends in Biotechnology (1997) 15:487-494), several kinds of insect cells (Vanden Broeck, Int. Rev. Cytology (1996) 164:189-268), amphibian cells (Jayawickreme et al., Current Opinion in Biotechnology (1997) 8: 629-634) and several mammalian cell lines (CHO, HEK293, COS, etc.; see Gerhardt, et al., Eur. J. Pharmacology (1997) 334:1-23). These examples do not preclude the use of other possible cell expression systems, including cell lines obtained from nematodes (PCT application WO 98/37177).

In one embodiment of the invention, methods of screening for antibodies which modulate the activity of M-CSF comprise contacting test antibodies with a M-CSF polypeptide and assaying for the presence of a complex between the antibody and the M-CSF. In such assays, the ligand is typically labeled. After suitable incubation, free ligand is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular antibody to bind to the M-CSF or M-CSFR polypeptide

In another embodiment of the invention, high throughput screening for antibody fragments or CDRs having suitable binding affinity to a M-CSF polypeptide is employed. Briefly, large numbers of different small peptide test compounds are synthesized on a solid substrate. The peptide test antibodies are contacted with a M-CSF polypeptide and washed. Bound M-CSF polypeptides are then detected by methods well known in the art. Purified polypeptides of the invention can also be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies can be

used to capture the protein and immobilize it on the solid support.

Combination Therapy

Having identified more than one M-CSF antibody that is effective in an animal model, it may be further advantageous to mix two or more such M-CSF antibodies together to provide still improved efficacy against cancer metastasis and/or bone loss associated with cancer metastasis. Compositions comprising one or more M-CSF antibody may be administered to persons or mammals suffering from, or predisposed to suffer from, cancer metastasis and/or bone loss associated with cancer metastasis.

Although M-CSF antibody therapy may be useful for all stages of cancers, antibody therapy may be particularly appropriate in advanced or metastatic cancers. Combining the antibody therapy method a chemotherapeutic or radiation regimen may be preferred in patients that have not received chemotherapeutic treatment, whereas treatment with the antibody therapy may be indicated for patients who have received one or more chemotherapies. Additionally, antibody therapy can also enable the use of reduced dosages of concomitant chemotherapy, particularly in patients that do not tolerate the toxicity of the chemotherapeutic agent very well.

The method of the invention contemplate the administration of single anti-M-CSF antibodies, as well as combinations, or "cocktails", of different antibodies. Such antibody cocktails may have certain advantages inasmuch as they contain antibodies which exploit different effector mechanisms or combine directly cytotoxic antibodies with antibodies that rely on immune effector functionality. Such antibodies in combination may exhibit synergistic therapeutic effects. In addition, the administration of anti-M-CSF and anti-M-CSFR antibodies may be combined with other therapeutic agents and/or procedures, including but not limited to various chemotherapeutic agents, androgen-blockers, and immune modulators (e.g., IL-2, GM-CSF), Bisphosphonate(s) (e.g., Aredia; Zometa; Clodronate), surgery, radiation, cytotoxic chemotherapy, hormone therapy (e.g., Tamoxifen; anti-Androgen therapy), antibody therapy (e.g., RANKL/RANK neutralizing antibodies; PTHrP neutralizing antibody, anti-Her2 antibody, VEGF neutralizing antibody), therapeutic protein therapy (e.g., soluble RANKL receptor; OPG, and PDGF and MMP inhibitors), small molecule drug therapy (e.g., Src-kinase inhibitor), kinase inhibitors of growth factor receptors; oligonucleotides therapy (e.g., RANKL or RANK or PTHrP Anti-sense), gene therapy (e.g., RANKL or RANK inhibitors), peptide therapy (e.g. muteins of RANKL) as

well as those proteins, peptides, compounds, and small molecules described herein.

A cytotoxic agent refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.*, I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin or synthetic toxins, or fragments thereof. A non-cytotoxic agent refers to a substance that does not inhibit or prevent the function of cells and/or does not cause destruction of cells. A non-cytotoxic agent may include an agent that can be activated to be cytotoxic. A non-cytotoxic agent may include a bead, liposome, matrix or particle (*see*, *e.g.*, U.S. Patent Publications 2003/0028071 and 2003/0032995 which are incorporated by reference herein). Such agents may be conjugated, coupled, linked or associated with an antibody according to the invention.

Cancer chemotherapeutic agents include, without limitation, alkylating agents, such as carboplatin and cisplatin; nitrogen mustard alkylating agents; nitrosourea alkylating agents, such as carmustine (BCNU); antimetabolites, such as methotrexate; folinic acid; purine analog antimetabolites, mercaptopurine; pyrimidine analog antimetabolites, such as fluorouracil (5-FU) and gemcitabine (Gemzar®); hormonal antineoplastics, such as goserelin, leuprolide, and tamoxifen; natural antineoplastics, such as aldesleukin, interleukin-2, docetaxel, etoposide (VP-16), interferon alfa, paclitaxel (Taxol®), and tretinoin (ATRA); antibiotic natural antineoplastics, such as bleomycin, dactinomycin, daunorubicin, doxorubicin, daunomycin and mitomycins including mitomycin C; and vinca alkaloid natural antineoplastics, such as vinblastine, vincristine, vindesine; hydroxyurea; aceglatone, adriamycin, ifosfamide, enocitabine, epitiostanol, aclarubicin, ancitabine, nimustine, procarbazine hydrochloride, carboquone, carboplatin, carmofur, chromomycin A3, antitumor polysaccharides, antitumor platelet factors, cyclophosphamide (Cytoxin®), Schizophyllan, cytarabine (cytosine arabinoside), dacarbazine, thioinosine, thiotepa, tegafur, dolastatins, dolastatin analogs such as auristatin, CPT-11 (irinotecan), mitozantrone, vinorelbine, teniposide, aminopterin, carminomycin, esperamicins (See, e.g., U.S. Patent No. 4,675,187), neocarzinostatin, OK-432, bleomycin, furtulon, broxuridine, busulfan, honvan, peplomycin, bestatin (Ubenimex®), interferon- β, mepitiostane, mitobronitol, melphalan, laminin peptides, lentinan, Coriolus versicolor extract, tegafur/uracil, estramustine (estrogen/mechlorethamine).

Further, additional agents used as therapy for cancer patients include EPO, G-CSF, ganciclovir; antibiotics, leuprolide; meperidine; zidovudine (AZT); interleukins 1

through 18, including mutants and analogues; interferons or cytokines, such as interferons α, β, and γ hormones, such as luteinizing hormone releasing hormone (LHRH) and analogues and, gonadotropin releasing hormone (GnRH); growth factors, such as transforming growth factor- β (TGF- β), fibroblast growth factor (FGF), nerve growth factor (NGF), growth hormone releasing factor (GHRF), epidermal growth factor (EGF), fibroblast growth factor homologous factor (FGFHF), hepatocyte growth factor (HGF), and insulin growth factor (IGF); tumor necrosis factor- α & β (TNF- α & β); invasion inhibiting factor-2 (IIF-2); bone morphogenetic proteins 1-7 (BMP 1-7); somatostatin; thymosin- α -1; γ-globulin; superoxide dismutase (SOD); complement factors; anti-angiogenesis factors; antigenic materials; and pro-drugs.

Prodrug refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic or non-cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into an active or the more active parent form. *See, e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy" *Biochemical Society Transactions*, 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella et al., "Prodrugs: A Chemical Approach to Targeted Drug Delivery," Directed Drug Delivery, Borchardt et al., (ed.), pp. 247-267, Humana Press (1985). Prodrugs include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use herein include, but are not limited to, those chemotherapeutic agents described above.

Administration and preparation

The anti-M-CSF antibodies used in the practice of a method of the invention may be formulated into pharmaceutical compositions comprising a carrier suitable for the desired delivery method. Suitable carriers include any material which, when combined with the anti-M-CSF antibodies, retains the anti-tumor function of the antibody and is nonreactive with the subject's immune systems. Examples include, but are not limited to, any of a number of standard pharmaceutical carriers such as sterile phosphate buffered saline solutions, bacteriostatic water, and the like. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like, and may include other proteins

for enhanced stability, such as albumin, lipoprotein, globulin, etc., subjected to mild chemical modifications or the like.

Therapeutic formulations of the antibody are prepared for storage by mixing the antibody having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide an immunosuppressive agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980).

The formulations to be used for in vivo administration must be sterile. This is

readily accomplished by filtration through sterile filtration membranes.

The antibody is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local treatment, intralesional administration. Parenteral infusions include intravenous, intraarterial, intraperitoneal, intramuscular, intradermal or subcutaneous administration. In addition, the antibody is suitably administered by pulse infusion, particularly with declining doses of the antibody. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic. Other administration methods are contemplated, including topical, particularly transdermal, transmucosal, rectal, oral or local administration e.g. through a catheter placed close to the desired site.

Compositions of the present invention can be in the form of, for example, granules, powders, tablets, capsules, syrup, suppositories, injections, emulsions, elixirs, suspensions or solutions. The instant compositions can be formulated for various routes of administration, for example, by oral administration, by nasal administration, by rectal administration, subcutaneous injection, intravenous injection, intramuscular injections, or intraperitoneal injection. The following dosage forms are given by way of example and should not be construed as limiting the instant invention.

For oral, buccal, and sublingual administration, powders, suspensions, granules, tablets, pills, capsules, gelcaps, and caplets are acceptable as solid dosage forms. These can be prepared, for example, by mixing one or more compounds of the instant invention, or pharmaceutically acceptable salts or tautomers thereof, with at least one additive such as a starch or other additive. Suitable additives are sucrose, lactose, cellulose sugar, mannitol, maltitol, dextran, starch, agar, alginates, chitins, chitosans, pectins, tragacanth gum, gum arabic, gelatins, collagens, casein, albumin, synthetic or semi-synthetic polymers or glycerides. Optionally, oral dosage forms can contain other ingredients to aid in administration, such as an inactive diluent, or lubricants such as magnesium stearate, or preservatives such as paraben or sorbic acid, or anti-oxidants such as ascorbic acid, tocopherol or cysteine, a disintegrating agent, binders, thickeners, buffers, sweeteners, flavoring agents or perfuming agents. Tablets and pills may be further treated with suitable coating materials known in the art.

Liquid dosage forms for oral administration may be in the form of

pharmaceutically acceptable emulsions, syrups, elixirs, suspensions, and solutions, which may contain an inactive diluent, such as water. Pharmaceutical formulations and medicaments may be prepared as liquid suspensions or solutions using a sterile liquid, such as, but not limited to, an oil, water, an alcohol, and combinations of these. Pharmaceutically suitable surfactants, suspending agents, emulsifying agents, may be added for oral or parenteral administration.

As noted above, suspensions may include oils. Such oil include, but are not limited to, peanut oil, sesame oil, cottonseed oil, corn oil and olive oil. Suspension preparation may also contain esters of fatty acids such as ethyl oleate, isopropyl myristate, fatty acid glycerides and acetylated fatty acid glycerides. Suspension formulations may include alcohols, such as, but not limited to, ethanol, isopropyl alcohol, hexadecyl alcohol, glycerol and propylene glycol. Ethers, such as but not limited to, poly(ethyleneglycol), petroleum hydrocarbons such as mineral oil and petrolatum; and water may also be used in suspension formulations.

For nasal administration, the pharmaceutical formulations and medicaments may be a spray or aerosol containing an appropriate solvent(s) and optionally other compounds such as, but not limited to, stabilizers, antimicrobial agents, antioxidants, pH modifiers, surfactants, bioavailability modifiers and combinations of these. A propellant for an aerosol formulation may include compressed air, nitrogen, carbon dioxide, or a hydrocarbon based low boiling solvent.

Injectable dosage forms generally include aqueous suspensions or oil suspensions which may be prepared using a suitable dispersant or wetting agent and a suspending agent. Injectable forms may be in solution phase or in the form of a suspension, which is prepared with a solvent or diluent. Acceptable solvents or vehicles include sterilized water, Ringer's solution, or an isotonic aqueous saline solution. Alternatively, sterile oils may be employed as solvents or suspending agents. Preferably, the oil or fatty acid is non-volatile, including natural or synthetic oils, fatty acids, mono-, di- or tri-glycerides.

For injection, the pharmaceutical formulation and/or medicament may be a powder suitable for reconstitution with an appropriate solution as described above. Examples of these include, but are not limited to, freeze dried, rotary dried or spray dried powders, amorphous powders, granules, precipitates, or particulates. For injection, the formulations may optionally contain stabilizers, pH modifiers, surfactants, bioavailability modifiers and

combinations of these.

For rectal administration, the pharmaceutical formulations and medicaments may be in the form of a suppository, an ointment, an enema, a tablet or a cream for release of compound in the intestines, sigmoid flexure and/or rectum. Rectal suppositories are prepared by mixing one or more compounds of the instant invention, or pharmaceutically acceptable salts or tautomers of the compound, with acceptable vehicles, for example, cocoa butter or polyethylene glycol, which is present in a solid phase at normal storing temperatures, and present in a liquid phase at those temperatures suitable to release a drug inside the body, such as in the rectum. Oils may also be employed in the preparation of formulations of the soft gelatin type and suppositories. Water, saline, aqueous dextrose and related sugar solutions, and glycerols may be employed in the preparation of suspension formulations which may also contain suspending agents such as pectins, carbomers, methyl cellulose, hydroxypropyl cellulose or carboxymethyl cellulose, as well as buffers and preservatives.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919), copolymers of L-glutamic acid and y ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S--S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

The formulations of the invention may be designed to be short-acting, fast-

releasing, long-acting, or sustained-releasing as described herein. Thus, the pharmaceutical formulations may also be formulated for controlled release or for slow release.

The instant compositions may also comprise, for example, micelles or liposomes, or some other encapsulated form, or may be administered in an extended release form to provide a prolonged storage and/or delivery effect. Therefore, the pharmaceutical formulations and medicaments may be compressed into pellets or cylinders and implanted intramuscularly or subcutaneously as depot injections or as implants such as stents. Such implants may employ known inert materials such as silicones and biodegradable polymers.

Besides those representative dosage forms described above, pharmaceutically acceptable excipients and carries are generally known to those skilled in the art and are thus included in the instant invention. Such excipients and carriers are described, for example, in "Remingtons Pharmaceutical Sciences" Mack Pub. Co., New Jersey (1991), which is incorporated herein by reference.

Specific dosages may be adjusted depending on conditions of disease, the age, body weight, general health conditions, sex, and diet of the subject, dose intervals, administration routes, excretion rate, and combinations of drugs. Any of the above dosage forms containing effective amounts are well within the bounds of routine experimentation and therefore, well within the scope of the instant invention.

M-CSF antibodies useful as therapeutics for cancer metastasis or bone loss associated with cancer metastasis will often be prepared substantially free of other naturally occurring immunoglobulins or other biological molecules. Preferred M-CSF antibodies will also exhibit minimal toxicity when administered to a mammal afflicted with, or predisposed to suffer from, cancer metastasis and/or bone loss associated with cancer metastasis.

The compositions of the invention may be sterilized by conventional, well known sterilization techniques. The resulting solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride and stabilizers (e.g., 1 20% maltose, etc.).

The M-CSF antibodies of the present invention may also be administered via

liposomes, which are small vesicles composed of various types of lipids and/or phospholipids and/or surfactant which are useful for delivery of a drug (such as the antibodies disclosed herein and, optionally, a chemotherapeutic agent). Liposomes include emulsions, foams, micelles, insoluble monolayers, phospholipid dispersions, lamellar layers and the like, and can serve as vehicles to target the M-CSF antibodies to a particular tissue as well as to increase the half life of the composition. A variety of methods are available for preparing liposomes, as described in, e.g., U.S. Patent Nos. 4,837,028 and 5,019,369, which patents are incorporated herein by reference.

Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein et al., Proc. Natl. Acad. Sci. USA 82: 3688 (1985); Hwang et al., Proc. Natl Acad. Sci. USA 77: 4030 (1980); and U.S. Patent Nos. 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556. Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin et al., J. Biol. Chem. 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome [*see, e.g.*, Gabizon et al., J. National Cancer Inst. 81(19): 1484 (1989)].

The concentration of the M-CSF antibody in these compositions can vary widely, i.e., from less than about 10%, usually at least about 25% to as much as 75% or 90% by weight and will be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Actual methods for preparing orally, topically and parenterally administrable compositions will be known or apparent to those skilled in the art and are described in detail in, for example, Remington's Pharmaceutical Science, 19th ed., Mack Publishing Co., Easton, PA (1995), which is incorporated herein by reference.

Determination of an effective amount of a composition of the invention to treat cancer metastasis and/or bone loss associated with cancer metastasis in a patient can be accomplished through standard empirical methods which are well known in the art. For example, the in vivo neutralizing activity of sera from a subject treated with a given dosage of M-CSF antibody may be evaluated using an assay that determines the ability of the sera to

block M-CSF induced proliferation and survival of murine monocytes (CD11b+ cell, a subset of CD11 cells, which expresses high levels of receptor to M-CSF) in vitro as described in Cenci et al., J Clin. Invest. 1055: 1279-87, 2000.

Compositions of the invention are administered to a mammal already suffering from, or predisposed to, cancer metastasis and/or bone loss associated with cancer metastasis in an amount sufficient to prevent or at least partially arrest the development of cancer metastasis and/or bone loss associated with cancer metastasis. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Effective amounts of a M-CSF antibody will vary and depend on the severity of the disease and the weight and general state of the patient being treated, but generally range from about 1.0 μg/kg to about 100 mg/kg body weight, or about 10 μg/kg to about 30 mg/kg, with dosages of from about 0.1 mg/kg to about 10 mg/kg or about 1 mg/kg to about 10 mg/kg per application being more commonly used. For example, about 10 μg/kg to 5 mg/kg or about 30 μg/kg to 1 mg/kg of antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. Administration is daily, on alternating days, weekly or less frequently, as necessary depending on the response to the disease and the patient's tolerance of the therapy. Maintenance dosages over a longer period of time, such as 4, 5, 6, 7, 8, 10 or 12 weeks or longer may be needed until a desired suppression of disease symptoms occurs, and dosages may be adjusted as necessary. The progress of this therapy is easily monitored by conventional techniques and assays.

Single or multiple administrations of the compositions can be carried out with the dose levels and pattern being selected by the treating physician. For the prevention or treatment of disease, the appropriate dosage of antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibody is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the antibody, and the discretion of the attending physician. The antibody is suitably administered to the patient at one time or over a series of treatments.

In any event, the formulations should provide a quantity of M-CSF antibody over time that is sufficient to effectively prevent or minimize the severity of cancer metastasis and/or bone loss associated with cancer metastasis. The compositions of the present invention may be administered alone or as an adjunct therapy in conjunction with other therapeutics known in the art for the treatment of cancer metastasis and/or bone loss associated with cancer metastasis.

The antibody composition will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The therapeutically effective amount of the antibody to be administered will be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat the M-CSF mediated disease, condition or disorder, particularly to treat cancer cells, and most particularly to treat tumor cell metastases. Such amount is preferably below the amount that is toxic to the host or renders the host significantly more susceptible to infections.

The antibody need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder in question. For example, in cancer, the antibody may be given in conjunction with chemo therapeutic agent or in ADEPT as described above. The effective amount of such other agents depends on the amount of antibody present in the formulation, the type of disease, condition or disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the diseases, disorders or conditions described above is provided, including for treatment of cancer. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is the antibody of the invention. The label on, or associated with, the container indicates that the composition is used for treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with

instructions for use.

Immunotherapy

Anti-M-CSF antibodies useful in treating cancers include those which are capable of initiating a potent immune response against the tumor and those which are capable of direct cytotoxicity. In this regard, anti-M-CSF antibodies may elicit tumor cell lysis by either complement-mediated or antibody-dependent cell cytotoxicity (ADCC) mechanisms, both of which require an intact Fc portion of the immunoglobulin molecule for interaction with effector cell Fc receptor sites or complement proteins. In addition, anti-M-CSF antibodies that exert a direct biological effect on tumor growth are useful in the practice of the invention. Potential mechanisms by which such directly cytotoxic antibodies may act include inhibition of cell growth, modulation of cellular differentiation, modulation of tumor angiogenesis factor profiles, and the induction of apoptosis. The mechanism by which a particular anti-M-CSF antibody exerts an anti-tumor effect may be evaluated using any number of *in vitro* assays designed to determine ADCC, ADMMC, complement-mediated cell lysis, and so forth, as is generally known in the art.

In one embodiment, immunotherapy is carried out using antibodies that have a higher affinity for the membrane-bound form of M-CSF (M-CSFα) than for the secreted forms of M-CSF. For example, antibodies may be prepared that specifically bind at or around the cleavage site of M-CSFα or to the portion of M-CSFα adjacent to the membrane. Such antibodies may also beneficially inhibit cleavage and release of the soluble active portion of M-CSFα.

The anti-M-CSF antibodies may be administered in their "naked" or unconjugated form, or may have therapeutic agents conjugated to them. In one embodiment, anti-M-CSF antibodies are used as a radiosensitizer. In such embodiments, the anti-M-CSF antibodies are conjugated to a radiosensitizing agent. The term "radiosensitizer," as used herein, is defined as a molecule, preferably a low molecular weight molecule, administered to animals in therapeutically effective amounts to increase the sensitivity of the cells to be radiosensitized to electromagnetic radiation and/or to promote the treatment of diseases that are treatable with electromagnetic radiation. Diseases that are treatable with electromagnetic radiation include neoplastic diseases, benign and malignant tumors, and cancerous cells.

The terms "electromagnetic radiation" and "radiation" as used herein include, but are not limited to, radiation having the wavelength of 10^{-20} to 100 meters. Preferred

embodiments of the present invention employ the electromagnetic radiation of: gamma-radiation (10^{-20} to 10^{-13} m), X-ray radiation (10^{-12} to 10^{-9} m), ultraviolet light (10 nm to 400 nm), visible light (400 nm to 700 nm), infrared radiation (700 nm to 1.0 mm), and microwave radiation (1 mm to 30 cm).

Radiosensitizers are known to increase the sensitivity of cancerous cells to the toxic effects of electromagnetic radiation. Many cancer treatment protocols currently employ radiosensitizers activated by the electromagnetic radiation of X-rays. Examples of X-ray activated radiosensitizers include, but are not limited to, the following: metronidazole, misonidazole, desmethylmisonidazole, pimonidazole, etanidazole, nimorazole, mitomycin C, RSU 1069, SR 4233, EO9, RB 6145, nicotinamide, 5-bromodeoxyuridine (BUdR), 5-iododeoxyuridine (IUdR), bromodeoxycytidine, fluorodeoxyuridine (FUdR), hydroxyurea, cisplatin, and therapeutically effective analogs and derivatives of the same.

Photodynamic therapy (PDT) of cancers employs visible light as the radiation activator of the sensitizing agent. Examples of photodynamic radiosensitizers include the following, but are not limited to: hematoporphyrin derivatives, Photofrin(r), benzoporphyrin derivatives, NPe6, tin etioporphyrin (SnET2), pheoborbide-a, bacteriochlorophyll-a, naphthalocyanines, phthalocyanines, zinc phthalocyanine, and therapeutically effective analogs and derivatives of the same.

In another embodiment, the antibody may be conjugated to a receptor (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a ligand (*e.g.*, avidin) which is conjugated to a cytotoxic agent (*e.g.*, a radionuclide).

The present invention further provides the above-described antibodies in detectably labeled form. Antibodies can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, etc.) fluorescent or luminescent or bioluminescent labels (such as FITC or rhodamine, etc.), paramagnetic atoms, and the like. Procedures for accomplishing such labeling are well known in the art; for example, see (Sternberger, L.A. et al., J. Histochem. Cytochem. 18:315 (1970); Bayer, E.A. et al., Meth. Enzym. 62:308 (1979); Engval, E. et al., Immunol. 109:129 (1972); Goding, J.W. J. Immunol. Meth. 13:215 (1976)).

"Label" refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody. The label may itself be detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. Alternatively, the label may not be detectable on its own but may be an element that is bound by another agent that is detectable (e.g. an epitope tag or one of a binding partner pair such as biotin-avidin, etc.) Thus, the antibody may comprise a label or tag that facilitates its isolation, and methods of the invention to identify antibodies include a step of isolating the M-CSF /antibody through interaction with the label or tag.

Exemplary therapeutic immunoconjugates comprise the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.*, an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate). Fusion proteins are described in further detail below.

Production of immunconjugates is described in U.S. Patent No. 6,306,393. Immnunoconjugates can be prepared by indirectly conjugating a therapeutic agent to an antibody component. General techniques are described in Shih et al., Int. J. Cancer 41:832-839 (1988); Shih et al., Int. J. Cancer 46:1101-1106 (1990); and Shih et al., U.S. Pat. No. 5,057,313. The general method involves reacting an antibody component having an oxidized carbohydrate portion with a carrier polymer that has at least one free amine function and that is loaded with a plurality of drug, toxin, chelator, boron addends, or other therapeutic agent. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate.

The carrier polymer is preferably an aminodextran or polypeptide of at least 50 amino acid residues, although other substantially equivalent polymer carriers can also be used. Preferably, the final immunoconjugate is soluble in an aqueous solution, such as mammalian serum, for ease of administration and effective targeting for use in therapy. Thus, solubilizing functions on the carrier polymer will enhance the serum solubility of the final immunoconjugate. In particular, an aminodextran will be preferred.

The process for preparing an inmmunoconjugate with an aminodextran carrier typically begins with a dextran polymer, advantageously a dextran of average molecular weight of about 10,000-100,000. The dextran is reacted with an oxidizing agent to affect a

controlled oxidation of a portion of its carbohydrate rings to generate aldehyde groups. The oxidation is conveniently effected with glycolytic chemical reagents such as $NaIO_4$, according to conventional procedures.

The oxidized dextran is then reacted with a polyamine, preferably a diamine, and more preferably, a mono- or polyhydroxy diamine. Suitable amines include ethylene diamine, propylene diamine, or other like polymethylene diamines, diethylene triamine or like polyamines, 1,3-diamino-2-hydroxypropane, or other like hydroxylated diamines or polyamines, and the like. An excess of the amine relative to the aldehyde groups of the dextran is used to ensure substantially complete conversion of the aldehyde functions to Schiff base groups.

A reducing agent, such as $NaBH_4$, $NaBH_3$ CN or the like, is used to effect reductive stabilization of the resultant Schiff base intermediate. The resultant adduct can be purified by passage through a conventional sizing column to remove cross-linked dextrans.

Other conventional methods of derivatizing a dextran to introduce amine functions can also be used, e.g., reaction with cyanogen bromide, followed by reaction with a diamine.

The amninodextran is then reacted with a derivative of the particular drug, toxin, chelator, immunomodulator, boron addend, or other therapeutic agent to be loaded, in an activated form, preferably, a carboxyl-activated derivative, prepared by conventional means, *e.g.*, using dicyclohexylcarbodiimide (DCC) or a water soluble variant thereof, to form an intermediate adduct.

Alternatively, polypeptide toxins such as pokeweed antiviral protein or ricin A-chain, and the like, can be coupled to aminodextran by glutaraldehyde condensation or by reaction of activated carboxyl groups on the protein with amines on the aminodextran.

Chelators for radiometals or magnetic resonance enhancers are well-known in the art. Typical are derivatives of ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). These chelators typically have groups on the side chain by which the chelator can be attached to a carrier. Such groups include, *e.g.*, benzylisothiocyanate, by which the DTPA or EDTA can be coupled to the amine group of a carrier. Alternatively, carboxyl groups or amine groups on a chelator can be coupled to a carrier by activation or prior derivatization and then coupling, all by well-known means.

Boron addends, such as carboranes, can be attached to antibody components

by conventional methods. For example, carboranes can be prepared with carboxyl functions on pendant side chains, as is well known in the art. Attachment of such carboranes to a carrier, e.g., aminodextran, can be achieved by activation of the carboxyl groups of the carboranes and condensation with amines on the carrier to produce an intermediate conjugate. Such intermediate conjugates are then attached to antibody components to produce therapeutically useful immunoconjugates, as described below.

A polypeptide carrier can be used instead of aminodextran, but the polypeptide carrier should have at least 50 amino acid residues in the chain, preferably 100-5000 amino acid residues. At least some of the amino acids should be lysine residues or glutamate or aspartate residues. The pendant amines of lysine residues and pendant carboxylates of glutamine and aspartate are convenient for attaching a drug, toxin, immunomodulator, chelator, boron addend or other therapeutic agent. Examples of suitable polypeptide carriers include polylysine, polyglutamic acid, polyaspartic acid, co-polymers thereof, and mixed polymers of these amino acids and others, e.g., serines, to confer desirable solubility properties on the resultant loaded carrier and immunoconjugate.

Conjugation of the intermediate conjugate with the antibody component is effected by oxidizing the carbohydrate portion of the antibody component and reacting the resulting aldehyde (and ketone) carbonyls with amine groups remaining on the carrier after loading with a drug, toxin, chelator, immunomodulator, boron addend, or other therapeutic agent. Alternatively, an intermediate conjugate can be attached to an oxidized antibody component via amine groups that have been introduced in the intermediate conjugate after loading with the therapeutic agent. Oxidation is conveniently effected either chemically, e.g., with $NaIO_4$ or other glycolytic reagent, or enzymatically, *e.g.*, with neuraminidase and galactose oxidase. In the case of an aminodextran carrier, not all of the amines of the aminodextran are typically used for loading a therapeutic agent. The remaining amines of aminodextran condense with the oxidized antibody component to form Schiff base adducts, which are then reductively stabilized, normally with a borohydride reducing agent.

Analogous procedures are used to produce other immunoconjugates according to the invention. Loaded polypeptide carriers preferably have free lysine residues remaining for condensation with the oxidized carbohydrate portion of an antibody component. Carboxyls on the polypeptide carrier can, if necessary, be converted to amines by, e.g., activation with DCC and reaction with an excess of a diamme.

The final immunoconjugate is purified using conventional techniques, such as sizing chromatography on Sephacryl S-300 or affinity chromatography using one or more CD84Hy epitopes.

Alternatively, immunoconjugates can be prepared by directly conjugating an antibody component with a therapeutic agent. The general procedure is analogous to the indirect method of conjugation except that a therapeutic agent is directly attached to an oxidized antibody component.

It will be appreciated that other therapeutic agents can be substituted for the chelators described herein. Those of skill in the art will be able to devise conjugation schemes without undue experimentation.

As a further illustration, a therapeutic agent can be attached at the hinge region of a reduced antibody component via disulfide bond formation. For example, the tetanus toxoid peptides can be constructed with a single cysteine residue that is used to attach the peptide to an antibody component. As an alternative, such peptides can be attached to the antibody component using a heterobifunctional cross-linker, such as N-succinyl 3-(2-pyridyldithio)proprionate (SPDP). Yu et al., Int. J. Cancer56:244 (1994). General techniques for such conjugation are well-known in the art. See, for example, Wong, Chemistry Of Protein Conjugation and Cross-Linking (CRC Press 1991); Upeslacis et al., "Modification of Antibodies by Chemical Methods," in Monoclonal Antibodies: Principles and Applications, Birch et al. (eds.), pages 187-230 (Wiley-Liss, Inc. 1995); Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in Monoclonal Antibodies: Production, Enineering and Clinical Application, Ritter et al. (eds.), pages 60-84 (Cambridge University Press 1995).

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-

methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionuclide to the antibody (*see, e.g.*, WO94/11026).

As described above, carbohydrate moieties in the Fc region of an antibody can be used to conjugate a therapeutic agent. However, the Fc region may be absent if an antibody fragment is used as the antibody component of the immunoconjugate. Nevertheless, it is possible to introduce a carbohydrate moiety into the light chain variable region of an antibody or antibody fragment. See, for example, Leung et al., J. Immunol. 154:5919 (1995); Hansen et al., U.S. Pat. No. 5,443,953. The engineered carbohydrate moiety is then used to attach a therapeutic agent.

In addition, those of skill in the art will recognize numerous possible variations of the conjugation methods. For example, the carbohydrate moiety can be used to attach polyethyleneglycol in order to extend the half-life of an intact antibody, or antigen-binding fragment thereof, in blood, lymph, or other extracellular fluids. Moreover, it is possible to construct a "divalent immunoconjugate" by attaching therapeutic agents to a carbohydrate moiety and to a free sulfhydryl group. Such a free sulfhydryl group may be located in the hinge region of the antibody component.

Anti-M-CSF Antibody Fusion Proteins

The present invention contemplates the use of fusion proteins comprising one or more anti-M-CSF antibody moieties and an immunomodulator or toxin moiety. Methods of making antibody fusion proteins are well known in the art. See, *e.g.*, U.S. Patent No. 6,306,393. Antibody fusion proteins comprising an interleukin-2 moiety are described by Boleti et al., Ann. Oncol. 6:945 (1995), Nicolet et al., Cancer Gene Ther. 2:161 (1995), Becker et al., Proc. Nat'l Acad. Sci. USA 93:7826 (1996), Hank et al., Clin. Cancer Res. 2:1951 (1996), and Hu et al., Cancer Res. 56:4998 (1996). In addition, Yang et al., Hum. Antibodies Hybridomas 6:129 (1995), describe a fusion protein that includes an $F(ab')_2$ fragment and a tumor necrosis factor alpha moiety.

Methods of making antibody-toxin fusion proteins in which a recombinant molecule comprises one or more antibody components and a toxin or chemotherapeutic agent also are known to those of skill in the art. For example, antibody-*Pseudomonas* exotoxin A fusion proteins have been described by Chaudhary et al., Nature 339:394 (1989), Brinkmann et al., Proc. Nat'l Acad. Sci. USA 88:8616 (1991), Batra et al., Proc. Nat'l Acad. Sci. USA 89:5867 (1992), Friedman et al., J. Immunol. 150:3054 (1993), Wels et al., Int. J. Can. 60:137

(1995), Fominaya et al., J. Biol. Chem. 271:10560 (1996), Kuan et al., Biochemistry 35:2872 (1996), and Schmidt et al., Int. J. Can. 65:538 (1996). Antibody-toxin fusion proteins containing a diphtheria toxin moiety have been described by Kreitman et al., Leukemia 7:553 (1993), Nicholls et al., J. Biol. Chem. 268:5302 (1993), Thompson et al., J. Biol. Chem. 270:28037 (1995), and Vallera et al., Blood 88:2342 (1996). Deonarain et al., Tumor Targeting 1:177 (1995), have described an antibody-toxin fusion protein having an RNase moiety, while Linardou et al., Cell Biophys. 24-25:243 (1994), produced an antibody-toxin fusion protein comprising a DNase I component. Gelonin was used as the toxin moiety in the antibody-toxin fusion protein of Wang et al., Abstracts of the 209th ACS National Meeting, Anaheim, Calif., Apr. 2-6, 1995, Part 1, BIOT005. As a further example, Dohlsten et al., Proc. Nat'l Acad. Sci. USA 91:8945 (1994), reported an antibody-toxin fusion protein comprising *Staphylococcal* enterotoxin-A.

Illustrative of toxins which are suitably employed in the preparation of such conjugates are ricin, abrin, ribonuclease, DNase I, Staphylococcal enterotoxin-A, pokeweed antiviral protein, gelonin, diphtherin toxin, Pseudomonas exotoxin, and Pseudomonas endotoxin. See, for example, Pastan et al., Cell 47:641 (1986), and Goldenberg, CA--A Cancer Journal for Clinicians 44:43 (1994). Other suitable toxins are known to those of skill in the art.

Antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a prodrug-activating enzyme which converts a prodrug (*e.g.*, a peptidyl chemotherapeutic agent, *See* WO81/01145) to an active anti-cancer drug. *See,* for example, WO88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for

converting prodrugs that contain D-amino acid substituents; carbohydrate-cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as abzymes, can be used to convert the prodrugs of the invention into free active drugs (*See, e.g.*, Massey, Nature 328: 457-458 (1987)). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (*See, e.g.*, Neuberger et al., Nature 312: 604-608 (1984))

Non-therapeutic uses

The antibodies of the invention may be used as affinity purification agents for M-CSF or in diagnostic assays for M-CSF protein, *e.g.*, detecting its expression in specific cells, tissues, or serum. The antibodies may also be used for in vivo diagnostic assays. Generally, for these purposes the antibody is labeled with a radionuclide (such as ^{111}In, ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^{3}H, ^{32}P or ^{35}S) so that the tumor can be localized using immunoscintiography.

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, such as ELISAs, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc. 1987). The antibodies may also be used for immunohistochemistry, to label tumor samples using methods known in the art.

As a matter of convenience, the antibody of the present invention can be provided in a kit, i.e., a packaged combination of reagents in predetermined amounts with instructions for performing the diagnostic assay. Where the antibody is labeled with an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.*, a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.*, a block buffer or lysis buffer) and

the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration.

The invention is illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLES

EXAMPLE 1

This example shows that M-CSF antibodies RX1 and 5A1 are species specific. RX1 is a commercially sold antibody that was available more than a year prior to the filing date of this application. Exemplary commercial sources include, but are not limited to, mouse anti-human M-CSF monoclonal antibody clones 116, 692, and 21 (Anogen); anti-human M-CSF antibody clones 21113.131, 26730, and 26786 (R & D Systems, Inc.); and anti-human M-CSF antibodyclone M16 (Antigenix America, Inc.).

To test the neutralizing activity of RX1 and 5A1, a proliferation assay of M-NFS-60 cell line was used (American Type Culture Collection Accession No. CRL-1838, available from ATCC in Rockville, MD, USA, derived from a myelogenous leukemia induced with the Cas-Br-MuLV wild mouse ecotropic retrovirous, responsive to both interleukin 3 and M-CSF and which contain a truncated c-myb proto-oncogene caused by the integration of a retrovirus). Proliferation of M-NFS-60 requires active M-CSF in a dose-dependent fashion. In the assay, M-NFS-60 cells were washed and plated in RPMI 1640 medium with 10% FBS and 3000 U/ml of M-CSF and 1% Pen/Strep. Recombinant human M-CSF (at 10 ng/ml final concentration), human or murine-specific, was incubated with various concentrations of antibodies for 1 hour at 37°C in 5% CO_2 in an incubator. Following the incubation, the mixture was added to the M-NFS-60 culture in 96 well microtiter plates. The total assay volume per well was 100µl, with 10 ng/ml M-CSF, and the antibody concentration indicated in Figure 5. Cells were incubated at 37 °C under 5% CO_2 for 72 hours before cell numbers were quantified by CellTiter Glo assay (Promega).

As shown in Figure 5, M-CSF antibodies RX1 and 5A1 are species specific. Cell proliferation is presented as the fluorescent reading from CellTiter Glo assay, which is

linear with cell number. Species specific neutralizing activity of RX1 and 5A1 is shown by its ability to inhibit M-NFS-60 in the presence of either human or murine M-CSF.

EXAMPLE 2

This example shows that antibody RX1 effectively inhibits osteolysis in a human xenograft model at a concentration 5mg/kg. Female nude mice at the age of 4-7 weeks old, average weight ~20g were be used in this study. Tumor cells (MDA-MB-231, $3x10^5$) suspended in 10µl of saline was be injected into the right tibia bone marrow cavity. Radiograms of the hind legs were taken one day after tumor inoculation for getting baseline image and checking for bone fracture caused by injection. Mice were randomly grouped into treatment groups including PBS and RX1 at 5 mg/kg, injected i.p. once a week for 6 weeks. At the end of study, radiograms of the hind legs were taken again and compared against baseline for bone damage. The degree of bone damaged caused by tumor was defined as shown in Figure 6. The group with RX1 5 mg/ml treatment showed statistically significant protection of the bone from tumor-cased damage.

EXAMPLE 3

This example shows that the number of metastases is reduced when antibody RX1 is administered to human breast cancer MDA-MB-231 bearing nude mice at a concentration of 5 mg/kg.

Female nude mice at the age of 4-7 weeks old, average weight ~20g were used for this study. Tumor cells (MDA-MB-231, $3x10^5$) suspended in 10µl of saline was injected into the right tibia bone marrow cavity. Radiograms of the hind legs were taken one day after tumor inoculation for getting baseline image and checking for bone fracture caused by injection. Mice were randomly grouped into the treatment groups including PBS and RX1 at 5 mg/kg injected i.p. once a week for 6 weeks. At the end of study, lungs of each treatment group were collected and fixed in Bouin's solution for metastatic lung nodule counting.

As shown in Figure 7, that the number of metastases is reduced when antibody RX1 is administered to human breast cancer MDA-MB-231 bearing nude mice at a concentration of 5 mg/kg.

EXAMPLE 4

This example sets out a procedure for humanization of the RX1 antibody.

Design of genes for humanized RX1 light and heavy chains

The nucleotide and amino acid sequence for murine RX1 are set forth in Figure 4B. The sequence of a human antibody identified using the National Biomedical Foundation Protein Identification Resource or similar database is used to provide the framework of the humanized antibody. To select the sequence of the humanized heavy chain, the murine RX1 heavy chain sequence is aligned with the sequence of the human antibody heavy chain. At each position, the human antibody amino acid is selected for the humanized sequence, unless that position falls in any one of four categories defined below, in which case the murine RX1 amino acid is selected:

(1) The position falls within a complementarity determining region (CDR), as defined by Kabat, J. Immunol., 125, 961-969 (1980);

(2) The human antibody amino acid is rare for human heavy chains at that position, whereas the murine RX1 amino acid is common for human heavy chains at that position;

(3) The position is immediately adjacent to a CDR in the amino acid sequence of the murine RX1 heavy chain; or

(4) 3-dimensional modeling of the murine RX1 antibody suggests that the amino acid is physically close to the antigen binding region.

To select the sequence of the humanized light chain, the murine RX1 light chain sequence is aligned with the sequence of the human antibody light chain. The human antibody amino acid is selected at each position for the humanized sequence, unless the position again falls into one of the categories described above and repeated below:

(1) CDR's;

(2) murine RX1 amino acid more typical than human antibody;

(3) Adjacent to CDR's; or

(4) Possible 3-dimensional proximity to binding region.

The actual nucleotide sequence of the heavy and light chain genes is selected as follows:

(1) The nucleotide sequences code for the amino acid sequences chosen as described above;

(2) 5' of these coding sequences, the nucleotide sequences code for a leader (signal) sequence. These leader sequences were chosen as typical of antibodies;

(3) 3' of the coding sequences, the nucleotide sequences are the sequences that follow the mouse light chain J5 segment and the mouse heavy chain J2 segment, which are part of the murine RX1 sequence. These sequences are included because they contain splice donor signals; and

(4) At each end of the sequence is an Xba I site to allow cutting at the Xba I sites and cloning into the Xba I site of a vector.

Construction of humanized light and heavy chain genes

To synthesize the heavy chain, four oligonucleotides are synthesized using an Applied Biosystems 380B DNA synthesizer. Two of the oligonucleotides are part of each strand of the heavy chain, and each oligonucleotide overlaps the next one by about 20 nucleotides to allow annealing. Together, the oligonucleotides cover the entire humanized heavy chain variable region with a few extra nucleotides at each end to allow cutting at the Xba I sites. The oligonucleotides are purified from polyacrylamide gels.

Each oligonucleotide is phosphorylated using ATP and T4 polynucleotide kinase by standard procedures (Maniatis et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). To anneal the phosphorylated oligonucleotides, they are suspended together in 40 ul of TA (33 mM Tris acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate) at a concentration of about 3.75 uM each, heated to 95 °C. for 4 min. and cooled slowly to 4 °C. To synthesize the complete gene from the oligonucleotides by synthesizing the opposite strand of each oligonucleotide, the following components are added in a final volume of 100 ul:

10 ul	annealed oligonucleotides
0.16 mM	each deoxyribonucleotide
0.5 mM	ATP
0.5 mM	DTT
100 ug/ml	BSA
3.5 ug/ml	T4 g43 protein (DNA polymerase)
25 ug/ml	T4 g44/62 protein (polymerase accessory protein)
25 ug/ml	45 protein (polymerase accessory protein)

The mixture is incubated at 37 °C for 30 min. Then 10 u of T4 DNA ligase is added and incubation at 37 °C is resumed for 30 min. The polymerase and ligase are inactivated by incubation of the reaction at 70 °C for 15 min. To digest the gene with Xba I, 50 ul of 2 X TA containing BSA at 200 ug/ml and DTT at 1 mM, 43 ul of water, and 50 u of Xba I in 5 ul is added to the reaction. The reaction is incubated for 3 hr at 37 °C, and then purified on a gel. The Xba I fragment is purified from a gel and cloned into the Xba I site of the plasmid pUC19 by standard methods. Plasmids are purified using standard techniques and sequenced using the dideoxy method.

Construction of plasmids to express humanized light and heavy chains is accomplished by isolating the light and heavy chain Xba I fragments from the pUC19 plasmid in which it had been inserted and then inserting it into the Xba I site of an appropriate expression vector which will express high levels of a complete heavy chain when transfected into an appropriate host cell.

Synthesis and affinity of humanized antibody

The expression vectors are transfected into mouse Sp2/0 cells, and cells that integrate the plasmids are selected on the basis of the selectable marker(s) conferred by the expression vectors by standard methods. To verify that these cells secreted antibody that binds to M-CSF, supernatant from the cells are incubated with cells that are known to express M-CSF. After washing, the cells are incubated with fluorescein-conjugated goat anti-human antibody, washed, and analyzed for fluorescence on a FACSCAN cytofluorometer.

For the next experiments, cells producing the humanized antibody are injected into mice, and the resultant ascites is collected. Humanized antibody is purified to substantial homogeneity from the ascites by passage through an affinity column of goat anti-human immunoglobulin antibody, prepared on an Affigel-10 support (Bio-Rad Laboratories, Inc., Richmond, Calif.) according to standard techniques. To determine the affinity of the humanized antibody relative to the original murine RX1 antibody, a competitive binding experiment is performed according to techniques known in the art.

EXAMPLE 4A

This example describes preparation of human engineered RX1 antibodies.

Design of human engineered sequences

Human engineering of antibody variable domains has been described by Studnicka [See, e.g., Studnicka et al. U.S. Patent No. 5,766,886; Studnicka et al. Protein Engineering 7: 805-814 (1994)] as a method for reducing immunogenicity while maintaining binding activity of antibody molecules. According to the method, each variable region amino acid has been assigned a risk of substitution. Amino acid substitutions are distinguished by one of three risk categories : (1) low risk changes are those that have the greatest potential for reducing immunogenicity with the least chance of disrupting antigen binding; (2) moderate risk changes are those that would further reduce immunogenicity, but have a greater chance of affecting antigen binding or protein folding; (3) high risk residues are those that are important for binding or for maintaining antibody structure and carry the highest risk that antigen binding or protein folding will be affected. Figure 4A shows the risk assignment for each amino acid residue of murine RX1 categorized as a high, moderate or low risk change.

Variable regions of the light and heavy chains of the murine RX1 antibody were human engineered using this method. Amino acid residues that are candidates for modification according to the method at low risk positions were identified by aligning the amino acid sequences of the murine variable regions with a human variable region sequence. Any human variable region can be used, including an individual VH or VL sequence or a human consensus VH or VL sequence. The amino acid residues at any number of the low risk positions, or at all of the low risk positions, can be changed. For the human engineered "low risk" heavy chain sequence in Figures 19A-B, human consensus Vh2 (based on Kabat) was used as the template, and for each position where the murine and human amino acid residues differed at low risk positions, an amino acid modification was introduced that replaced the murine residue with the human residue. For the human engineered "low risk" light chain sequence in Figures 20A-B, human consensus kappa 3 (based on Kabat) was used as the template, and for each position where the murine and human amino acid residues differed at low risk positions, an amino acid modification was introduced that replaced the murine residue with the human residue. A total of 16 amino acid modifications were made to the light chain and 8 modifications were made to the heavy chain.

Similarly, amino acid residues that are candidates for modification according to the method at all of the low and moderate risk positions were identified by aligning the amino acid sequences of the murine variable regions with a human variable region sequence. The amino acid residues at any number of the low or moderate risk positions, or at all of the low and moderate risk positions, can be changed. For the human engineered heavy chain

sequence in Figures 19A-B, human consensus Vh2 (based on Kabat) was used as the template, and for each position where the murine and human amino acid residues differed at low or moderate risk positions, an amino acid modification was introduced that replaced the murine residue with the human residue. For the human engineered light chain sequence in Figures 20A-B, human consensus kappa 3 (based on Kabat) was used as the template, and for each position where the murine and human amino acid residues differed at low or moderate risk positions, an amino acid modification was introduced that replaced the murine residue with the human residue. A total of 19 amino acid modifications were made to the light chain and 12 modifications were made to the heavy chain.

An "alternative low risk" light chain sequence was also prepared as shown in Figures 21A-B, in which the modification at position 54 was reversed back to murine. An "alternative low+moderate risk" light chain sequence was also prepared as shown in Figures 21A-B, in which the modifications at positions 54-56 were reversed back to murine.

Finally, human engineered "low risk" and "low+moderate risk" light chain sequences were also produced using human germline VK6 subgroup 2-1-(1) A14 as the template, as shown in Figures 22A-B.

Also contemplated by the present invention is retaining amino acids 41-43 (NGS) of Figure 4A which represent the glycosylation site. Alternatively, only one or two of amino acids 41-43 (e.g., NG) may be retained.

Preparation of Expression Vectors

DNA fragments encoding each of the above-described heavy and light chain sequences along with antibody-derived signal sequences were constructed using synthetic nucleotide synthesis of 6 overlapping oligonucleotides. These segments were annealed to each other, extended with DNA polymerase and then the assembled variable region amplified by PCR. DNA encoding each of the light chain amino acid sequences described above were inserted into vector pMPX10. DNA encoding each of the heavy chain amino acid sequences described above were inserted into vector pMPX6. These vectors contain a hCMV promoter and a mouse kappa light chain 3' untranslated region as well as selectable marker genes such as neo.

Vectors also were constructed for transient transfection. These vectors are similar to those described above for permanent transfections except that they contain the Epstein-Barr virus oriP instead of the neo gene. A vector comprising the desired human engineered light

plus heavy chain genes was then constructed. This vector contains the hCMV promoter, SV40 16S splice acceptor, light chain genomic DNA including polyA site, and a selectable marker gene such as neo or his and the ampicillin resistance gene. Vectors for the following heavy/light chain combinations were produced: heavy chain with low risk changes and light chain with low risk changes, heavy chain with low+moderate risk changes and light chain with low+moderate risk changes, heavy chain with low risk changes and light chain with low+moderate risk changes, heavy chain with low+moderate risk changes and light chain with low risk changes, heavy chain with low+moderate risk changes and alternative low+moderate risk light chain. Vectors comprising two copies of each light and heavy chain genes (four gene vectors) also can be constructed.

Development and characterization of transfected cell lines

Separate vectors each containing oriP from the Epstein-Barr Virus and the light chain or heavy chain genes are transfected transiently into 293E cells. Transiently transfected cells are allowed incubate for up to 10 days after which the supernatant is recovered and antibody purified using Protein A chromatography. The vectors described above containing one or two copies of the light and heavy genes together are transfected into Ex-Cell 301-adapted CHO-K1 cells. CHO-K1 cells adapted to suspension growth in Ex-Cell 301 medium are typically electroporated with 40 ug of linearized vector. The cells are plated in 96 well plates containing Ex-Cell 301 medium supplemented with 2% FBS and G418. Clones are screened in 96 well plates and the top 22 clones for each transfection are transferred to 24 well plates containing Ex-Cell 301 medium without FBS.

A productivity test is performed in 24 well plates in Ex-Cell 301 medium with or without 2% FBS. Cells are grown to extinction and culture supernatants tested for levels of secreted antibody by an immunoglobulin ELISA assay for IgG.

The top clones are transferred to shake flasks containing Ex-Cell 301 medium. As soon as the cells are adapted to suspension growth, a shake flask test is performed with these clones in Ex-Cell 301 medium with and without 2% FBS. The cells are grown for up to 10 days in 125 ml Erlenmeyer flasks containing 25 ml media. The flasks are sealed for most of the incubation period and the levels of immunoglobulin polypeptide in the culture medium are determined by IgG ELISA at the end of the incubation period. Multiple sequential transfections of the same cell line with two or three multi-unit transcription vectors results in clones and cell lines that exhibit further increases in levels of immunoglobulin production,

preferably to 200 ug/ml, 250 ug/ml, 300 ug/ml or more.

Binding activity

The MCSF binding activity of the recombinant human engineered antibodies is evaluated. Protein is purified from shake flask culture supernatants by passage over a protein A column followed by concentration determination by A_{280}. Binding assays are performed as described in Example 1 above or 12 below. Immulon II plates are precoated with the sM-CSF antigen pre-diluted in a PBS coating solution to immobilize it to the microplate. Various test concentrations of M-CSF ranging from 0.25 to 20 ug/ml are added at 50 ul/well and incubated at 4°C overnight. The plates are then washed 3 times with PBS-0.05% Tween. Blocking is performed by adding in PBS-0.05% Tween 1% BSA followed by a 30 minute incubation at 37°C. Dilutions of immunoglobulin polypeptides are prepared in PBS-0.05% Tween 1% BSA solution. 2- or 3-fold serial dilutions are prepared and added (100 ul/well) in duplicate or triplicate. After a 90 minute incubation at 37°C, the microplate is washed 3 times with PBS-0.05% Tween. For signal development, goat anti-human IgG (gamma- or Fc-specific) secondary antibody conjugated to peroxidase is added to each well and incubated for 60 minutes at 37°C followed by addition of OPD at 0.4 mg/ml in citrate buffer plus 0.012% H_2O_2. After 5 – 10 minutes at room temperature the assay is stopped by the addition of 100 ul 1M H_2SO_4 and the plates are read at 490nm. Both goat anti-human IgG (gamma-specific) and goat anti-human IgG (Fc-specific) antibodies have been employed.

Purification

A process for the purification of immunoglobulins polypeptides from vectors and all lines according to the invention may be designed. According to methods well known in the art, cells are removed by filtration after termination. The filtrate is loaded in multiple passes onto a Protein A column. The column is washed and then the expressed and secreted immunoglobulin polypeptides are eluted from the column. For preparation of antibody product, the Protein A pool is held at a low pH (pH 3 for a minimum of 30 minutes and a maximum of one hour) as a viral inactivation step. An adsorptive cation exchange step is next used to further purify the product. The eluate from the adsorptive separation column is passed through a virus retaining filter to provide further clearance of potential viral particles. The filtrate is further purified by passing through an anion exchange column in which the product does not bind. Finally, the purification process is concluded by transferring the product into the formulation buffer through diafiltration. The retentate is adjusted to a

protein concentration of 5 mg/mL and a stabilizer is added.

EXAMPLE 5

The following example sets out a procedure for the treatment of humans using M-CSF-specific antibody, such as an RX1-derived or RX1-competing antibody. The expected efficacious dosing range is 2 ug/kg to 10 mg/kg. This estimation is based on following rationale substantiated by experimental data:

The measured M-CSF level in human plasma (both healthy and breast cancer patients) is about 1 ng/ml. M-CSF neutralizing antibody RX1 has a measured EC_{50} of 2 ng/ml against 1 ng/ml human M-CSF. Accordingly, the effective antibody concentration in human plasma is expected to be 10 to 50,000 fold over its EC_{50}, i.e. 20 ng/ml to 100 ug/ml antibody in human plasma. Based on PK studies, in order to effectuate this concentration in human patients, a dosing of 2 μg/kg to 10 mg/kg is required to reach 20 ng/ml to 100 ug/ml antibody concentration in plasma.

EXAMPLE 6

This example sets out a procedure for the evaluation of the anti-cancer activity of anti-M-CSF monoclonal antibody in a subcutaneous model. Example 2 above showed that anti-M-CSF monoclonal antibody treatment significantly inhibited the tumor growth in bone marrow. The purpose of this study is to evaluate whether the antibody can also inhibit the tumor growth in soft tissue.

Female nu/nu mice at the age of 10 weeks old, average weight ~20g will be used for this study. Mice will undergo an acclimation period of at least 7 days prior to study start. On day 0, the right flank of nude mice will be injected with SW620 human colon cancer cells subcutaneously at $5x10^6$ cells per mouse per 100μl. When tumor volume reaches 100-200 mm^3 (usually 1 week after tumor inoculation), mice will be randomized into 5 groups at 10 mice per group as follows:

1) PBS
2) RX1
3) 5A1
4) mIgG1+rIgG1 isotype Ab control
5) 5A1+RX1

Mice will be treated intraperitoneally with the designated antibodies at 10mpk once a week for 4 weeks. When tumor volume reaches 2000 mm^3, the study will be terminated. Alternatively, animals will also be euthanized when any of the following situations are met: tumor surface ulceration is bigger than 30% of total tumor surface area, significant body weight loss (>20%), dehydration, and moribund. Whole blood will be collected from all of the mice and monocyte population will be analyzed as a potential surrogate marker. Tumor growth/size will be measured by 2-D analysis. Measurements of tumor width and length will be used to calculate tumor volume. It is expected that tumor growth in soft tissue will be inhibted as a result of the foregoing experiment.

EXAMPLE 7

The following example sets out a procedure for the evaluation of combination therapy for the treatment and prevention severe osteolytic disease associated with cancer metastasis.

Experimental Design. The study described in Example 5 above is repeated essentially as described with the following exceptions. In addition to the antibody or antibody combination set out in the treatment groups below, the animals will receive one of the following additional treatments:

1. Bisphosphonate (e.g., Aredia; Zometa; Clodronate).
2. Surgery
3. Radiation
4. Chemotherapy
5. Hormone therapy (e.g., Tamoxifen; anti-Androgen therapy)
6. Antibody therapy (e.g., RANKL/RANK neutralizing antibodies; PTHrP neutralizing antibody)
7. Therapeutic protein therapy (e.g., soluble RANKL receptor; OPG, and PDGF and MMP inhibitors)
8. Small molecule drug therapy (e.g., Src-kinase inhibitor)
9. Oligonucleotides therapy (e.g., RANKL or RANK or PTHrP Anti-sense)
10. Gene therapy (e.g., RANKL or RANK inhibitors)
11. Peptide therapy (e.g. muteins of RANKL)

The treatment groups are as follows. The above additional treatments are indicated below as "plus therapy X":

1. PBS only
2. treatment with therapy X only
3. rat IgG1 isotype control

4. murine IG1 isotype control
5. RX1 anti-human MCSF only
6. 5A1 rat IgG1 anti-mouse MCSF only
7. rat IgG1 and murine IgG1 isotype control combination
8. RX1 an 5A1 combination
9. rat IgG1 isotype control plus therapy X
10. murine IG1 isotype control plus therapy X
11. RX1 anti-human MCSF plus therapy X
12. 5A1 rat IgG1 anti-mouse MCSF plus therapy X
13. rat IgG1 and murine IgG1 isotype control combination plus therapy X
14. RX1 and 5A1 combination plus therapy X

Dosing: 0.1-30 mg/kg each antibody is used for administration to each animal. Preferred dosing is 10 mg/kg. The administration route can be IV, IP, SC. The preferred route is IP. Treatment will begin the day following injection of tumor cells, as described in Example 5, above.

Measurements. To assess the severity of osteolysis among the various treatment groups, each mouse receives a baseline Faxitron image taken the day following injection of tumor cells. A Faxitron image is also taken at the end of the study (8 weeks). Tumor growth is simultaneously measured using the Xenogen system since the tumor cells stably express luciferase. It is expected that combination therapy for the treatment and prevention severe osteolytic disease associated with cancer metastasis will be improved with relative to antibody therapy alone.

EXAMPLE 8

The following example provides a protocol for evaluating the ability of M-CSF-specific antibody to bind to, for example, breast cancer cells (cell line MDA231) or multiple myeloma cancer cells (cell line ARH77) using a fluorescence-activated cell sorter.

The cells were first washed twice with PBS (no Ca^{2+}, Mg^{2+}). For each 10-cm plate, 2ml of 3 mM EDTA was added, and the paltes were incubated at 37 °C for 2-3 minutes, until the cells were rounded and began to detach from the dish. Next, 10 ml of buffer A (PBS + 5% FBS) was added and mixed. At that time, the cells were pelleted and resuspended at about $5x10^6$ cells/ml in PBS+5% FBS, and the cells were placed into tubules at 100 µl/sample.

At this point, 0.1-10 ug/ml of the primary antibody (used at indicated

concentration of M-CSF antibody or control antibody) was added. Dilution, if necessary, was made in 5% FBS/PBS. The mixture was then incubated for 30 min at 4 °C. Following the incubation period, the cells were washed 3 times by centrifugation at 400 g for 5 min., and the cells were resuspended in PBS.

The FITC or PE-labeld anti-IgG antibody (0.25 ug/sample) was diluted in 1% BSA/PBS at the optimal dilution, and the cells were resuspended in this solution and incubated for 30 min at 4 °C. Next, the cells were washed 3 times as described above. Following th cell washes, the cells were resuspended with 0.5 ml/sample PI-PBS (if necessary to distinguish dead cells from live ones). The cells can also be fixed for later analysis (the cells can last about 3 days if they are fixed with 0.1% formaldehyde). The cells were next analyzed in a fluorescence-active FACS using standard procedures.

As shown in Figure 8A and 8B, an MCSF-specific antibody RX1 bound to breast cancer cell line MDA231 or to multiple myeloma cancer cell line ARH77 at a variety of antibody concentrations as indicated.

EXAMPLE 9

The following example shows M-CSF is prevalent on a number of cancer cell surfaces. Immunohistochemical staining of M-CSF was carried using a M-CSF-specific antibody RX1 was carried out as follows.

At the outset, slides were heated in anoven at 55 – 60°C for 1 hour and allowed to cool for 2-3 minutes. The following de-waxing and re-hydration parameters were used:

a. Xylene	3 x 5 minutes
b. 100% Reagent Alcohol	2 x 5 minutes
c. 95% Reagent Alcohol	2 x 4 minutes
d. 75% Reagent Alcohol	2 x 3 minutes
e. 50% Reagent Alcohol	1 x 3minutes
g. dI H2O	2 – 3 quick rinses

Prior to the peroxide blocking step, antigen retrieval was prepared using 1 x

Biogenex Citra Plus. The solution was initially microwaved at full power to boil. Once the solution boiled, the microwave was quickly set for another 13 min at power-level 2, and allowed to cool before proceeding. The peroxide blocking step was performed as follows. The slides were immersed slides in 3% H_2O_2 (25ml 30% to 250ml dI H_2O) and placed at room temperature for 10 minutes. The slides were next rinsed 2x with dI H_2O, and washed with 1 X PBS 2 x 2 minutes.

The avidin/biotin blocking procedure was performed as follows. Slides were palced flat on a metal rack. A Blue PAP pen was used (hydrophobic slide marker) around tissue. Next, 2 drops Zymed Avidin (Reagent A)---enough to cover tissue--was added and the slides were incuabated at room temperature for 10 min. Following the incubation, , the slides were washed as follows:

2 x 3 minute washes in 1 X PBS.

2 drops Zymed Biotin (Reagent B), room temperature for 10 min.

2 x 3 minute washes in 1 X PBS.

The protein block ing procedure was performed as follows. First, 10% serum [to 2% final concentration] of secondary antibody species was added. The BioGenex Power Block was next diluted to 1 X with dI H_2O. The rack of slides was immersed in Power Block for 8 min at room temperature, and the slides were rinsed 2x in 1X PBS.

For the addition of the primary antibody (RX1), the slides were placed flat on a metal rack. Antibody was added to cover each section (~350µl), and the antibody was spread with pipet tip (if necessary) without scraping tissue. The slides were then incubated for 1 hour at room temperature. Following the incubation, the slides were washed 3 x with 1 x PBS 3-5 minutes each time. At this point, BioGenex Multi-Link was applied to sections & incubated for 10-11 minutes at room temperature. The sections were then washed 3 minutes each time.

Labelling was performed by applying BioGenex HRP Label to sections, which were then incubated at room temperature for 10-11 min and washed with 1 x PBS 3 x 3 minutes. Next, BioGenex H_2O_2 substrate was added (1 drop AEC for every 2.5 ml H2O2) to the sections and incubated at room temperature for 10 min. The sections were then rinsed several times with dI H_2O. The counterstaining step was performed as follows. The sections were staine with hematoxylin for 1 minute at room temperature. Next, the sections were rinse with H_2O twice, and then incubated in 1 X PBS for 1 minute. Sections were then rinsed

well with H_2O to remove PBS. Sections were mounted by applying a drop of BioGenex Super Mount to the section section and then air drying over night at room temperature.

As shown in Figure 9, M-CSF is prevalent on a number of cancer cell surfaces. Sections for the indicated cancer cell types were scored as follows:

0	No staining
1	Staining was similar to background
2	Positive, but weak staining
3	Positive and significant staining
4	Positive and strong staining

EXAMPLE 10

The following example shows the procedure for producing antibodies MC1 and MC3. MC1 and MC3 are two monoclonal murine antibodies that neutralize human M-CSF antibody and bind to human M-CSF. The amino acid sequences of these antibodies are shown in Figures 14 and 15, respectively. They were identified by a series of steps including a) immunization of Balb C mice with recombinant human M-CSF; b) screening for positive clones that produce antibodies which bind to human M-CSF in an ELISA format; c) subcloning of positive clones to generate stable hybridoma clones; d) scale-up of cell culture to produce large quantity of antibodies; e) purification and characterization of antibodies in affinity analysis, cell binding, and neutralizing activity assay as described in previous examples.

Figures 16A and 16B show the alignment of the CDRs of the heavy and light chains, respectively, of antibodies RX1, 5H4 (sequence set forth in Figure 13), MC1 and MC3.

EXAMPLE 11

This example shows that M-CSF antibodies RX1 and 5H4, as well as Fab fragments thereof, have different neutralizing activities. The following example also shows that antibodies RX1, 5H4, and MC3 have varying affinities for M-CSF. This example further demonstrates that the affinities of the aforementioned intact antibodies are higher relative to Fab fragments of the aforementioned antibodies.

Neutralization activities of intact RX1 and 5H4 versus Fab fragments of RX1 and 5H4 were determined by measuring M-CSF-dependent cell proliferation in the presence

of various concentrations of antibody. The cell proliferation was determined by chemiluminescent dye. As shown in Figure 17, intact RX1 has the highest potency, while the Fab fragment of RX1 loses its potency and behaves like 5H4 and the 5H4 Fab fragment.

Binding properties of the aforementioned antibodies were analyzed using Biacore anlyses. In order to determine the relative affinities of RX1, 5H4, and MC3 to M-CSF, rabbit anti-mouse Fc was immobilized onto a CM5 biosensor chip via amine coupling (Rmax ~15). The aforementioned antibodies were then captured on the anti-mouse Fc/CM5 biosensor chip at 1.5µg/ml for 3 min at 2µl/min. MCSF was flowed over the modified biosensor surface at varying concentrations. Test antibodies and antigen were diluted in 0.01 M HEPES pH 7.4, 0.15 M NaCL, 3 mM EDTA, 0.005% Surfactant P20 (HBS-EP). All experiments were performed at 25°C. Kinetic and affinity constants were determined using Biaevaluation software (Biacore) with a 1:1 interaction model/global fit. As shown below in Table 2, RX1 binds to M-CSF with the highest affinity relative to 5H4 and MC3.

Table 2

	Ka (M-1 Sec-1)	Kd (sec-1)	KD (nM)
RX1	1.64e6	2.7e-4	0.16
5H4	5.94e5	1.77e-3	3.0
MC3	7.04e5	1.93e-4	0.27

To determine the relative differences in the binding affinity of intact Mab and Fab fragments of RX1, 5H4, and MC3, an alternate configuration was used in the Biacore analysis. Specifically, M-CSF was immobilized onto CM5 biosensor chip via amine coupling. 0.05µg/ml M-CSF in 10mM Na Acetate pH 4.0 was injected at 1 µl/min for 5 minutes to achieve RL=6RU. Test antibody (or Fab fragment) were flowed over the modified biosensor surface at varying concentrations. Test antibodies were diluted in 0.01 M HEPES pH 7.4, 0.15 M NaCL, 3 mM EDTA, 0.005% Surfactant P20 (HBS-EP) and all experiments were done at 25°C. Kinetic and affinity constants were determined using Biaevaluation software (Biacore) with a 1:1 interaction model/global fit. As shown below in Table 3, RX1 binds M-CSF with the highest affinity relative to the other antibodies tested. The Fab fragment of RX1 binds M-SCF with a significantly lower affinity relative to the RX1 holoprotein.

Table 3

	Ka (M-1 Sec-1)	Kd (sec-1)	KD (nM)
RX1	2.27e5	2.27e-4	1.0
RX1 Fab	2.77e5	3.14e-3	11.3
rRX1(mouse)	2.34e5	2.35e-4	1.0
rRX1 Fab (mouse)	2.81e5	3.03e-3	10.8
5H4	1.27e5	1.26e-3	9.9
5H4 Fab	2.04e5	2.85e-3	14.0
MC3	1.22e5	4.29e-4	3.5

The binding affinity and neutralization data indicate that the neutralization activity of RX1 is due primarily to its remarkably high affinity for M-CSF, and that this high affinity may be due at least in part to the ability of both arms of the antibody to bind the M-CSF dimer simultaneously.

EXAMPLE 12

The following example reveals the linear epitope (i.e., amino acid sequence) on M-CSF recognized by antibodies RX1, 5H4, and MC3.

Initially, the epitope mapping strategy was designed to determine whether antibodies RX1, 5H4, and MC3 recognized linear epitopes or conformational epitopes within M-CSF. Accordingly, the anti-M-CSF antibodies were tested against 0.1μg M-CSF under reducing as well as non-reducing conditions. Only the the non-reduced form of M-CSF was recognized by each of the antibodies, suggesting the epitopes recognized are discontinuous in nature.

Next, the linear epitope of M-CSF was determined for each antibody. Specifically, SPOTs membranes (Sigma Genosys) were prepared where the M-CSF fragment sequence of interest, overlapping 10mer peptides synthesized with one amino acid offset, were loaded onto the cellulose membrane support. These membranes were then probed with the aforementioned antibodies and reactive SPOTs were identified. The peptide sequence was then identified by its corresponding location on the membrane, and overlapping amino acids within the positive reacting peptides were identified as the epitope. As shown in Figure 18, RX1 binds to a different linear epitope than 5H4 and MC3, which map to a different location on M-CSF. RX1 binds to a linear epitope represented by RFRDNTAN, amino acids

98-105 of M-CSF of Figure 12. 5H4 binds to a linear epitope represented by ITFEFVDQE, amino acids 65-73 of M-CSF of Figure 12. MC3 binds to two linear epitopes represented by (1) ITFEFVDQE, amino acids 65-73 of M-CSF of Figure 12 and (2) FYETPLQ, amino acids 138-144 of M-CSF of Figure 12.

All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention.

ABSTRACT

M-CSF-specific antibody RX1 is provided, along with pharmaceutical compositions containing antibody RX1, kits containing a pharmaceutical composition, and methods of preventing and treating bone loss in a subject afflicted with an osteolytic disease.

5

FIG. 1

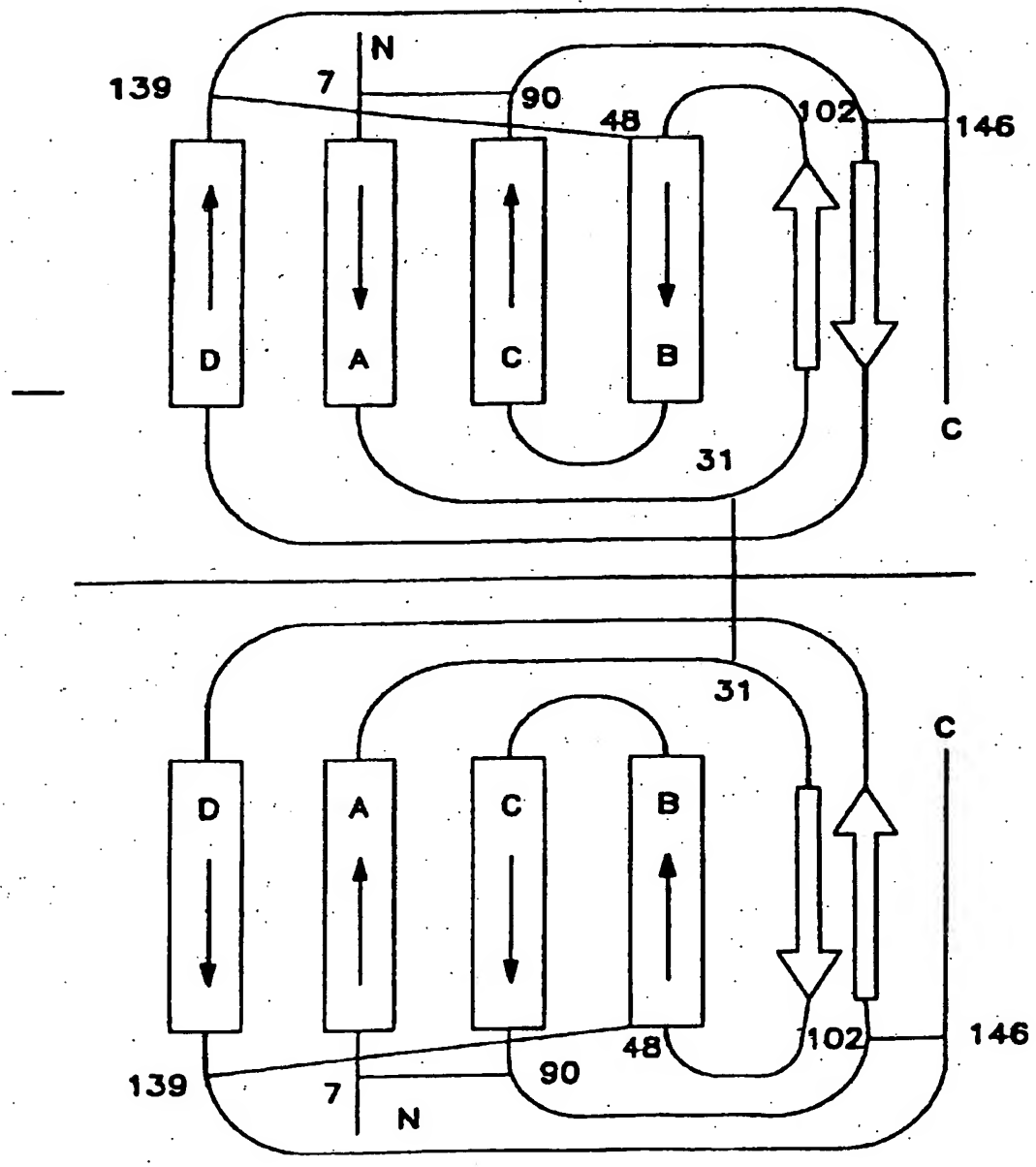

FIG. 2

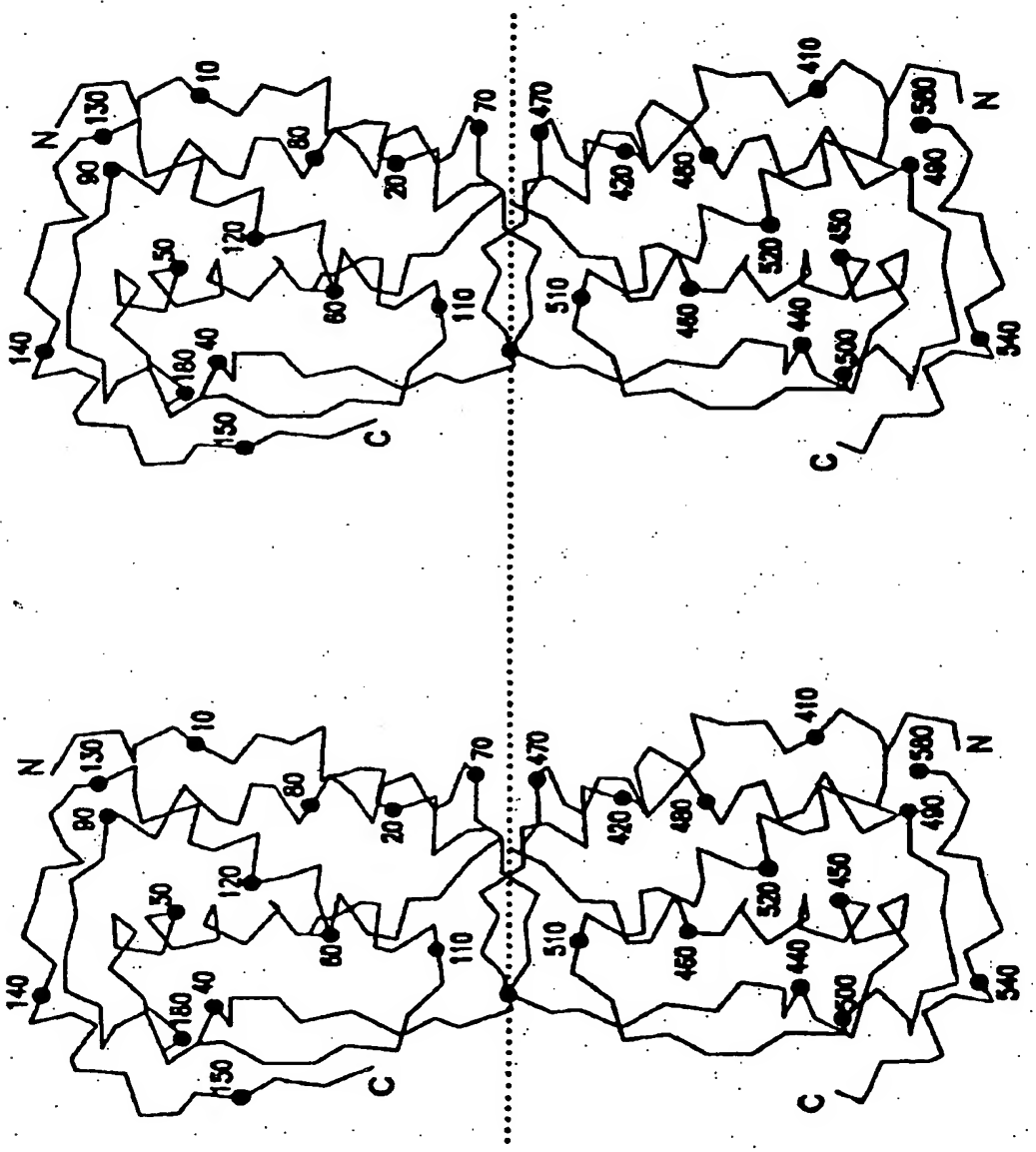
N
C
10
130
80
70
20
120
50
60
110
140
40
180
150
410
470
420
480
490
500
450
520
510
460
440
530
540

FIG. 3

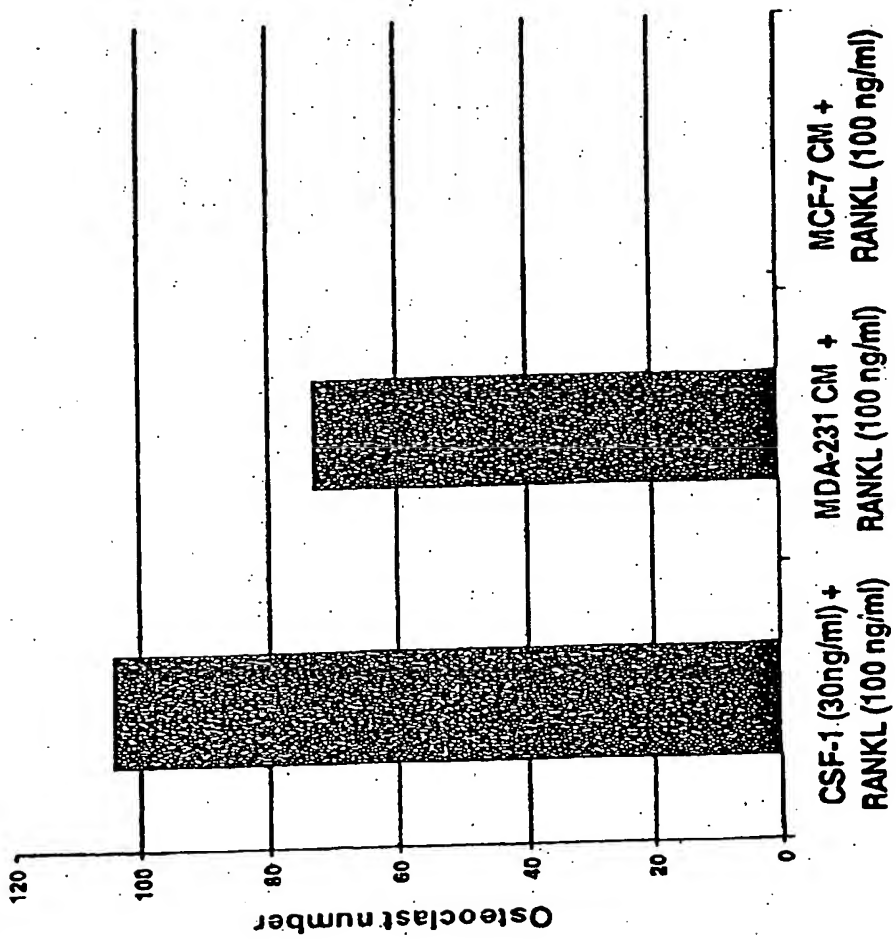
Osteoclast number
120
100
80
60
40
20
0
CSF-1 (30ng/ml) + RANKL (100 ng/ml)
MDA-231 CM + RANKL (100 ng/ml)
MCF-7 CM + RANKL (100 ng/ml)

Fig. 4A

RX1 Light chain amino acid sequence:

1 2 3

DILLTQSPAILSVSPGERVSFSC**RASQSIGTSIH**WYQQRTNGSPRLLIK**YASESIS**GIPSRFSGSGSGTDFTLSINSVESEDIADYYC**QQINSWPTT**FGGGTKLEIKRADAAPTVSIFPPSSE
QLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

RX1 Heavy Chain amino acid sequence:

4 5 6

DVQLQESGPGLVKPSQSLSLTCTVTDYSIT**SDYAWN**WIRQFPGNKLEWMG**YISYSGSTSYNPSLKS**RISITRDTSKNQFFLQLNSVTTEDTATYYCAS**FDYAHAMDY**WGQGTSVTVSSAKTTA
PSVYPLAPVCGDTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRGPTIKPCPPCKCPAPNLLGGPSVFIFPP
KIKDVLMISLSPIVTCVVVDVSEDDPDVQISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIERTISKPKGSVRAPQVYVLPPpEEEMTKKQVTLTCM
VTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSDGSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHhttKSFSRTPG

RX1 heavy chain nucleotide sequence:

atgggttggtcctgtatcatcctattcctggtggccactgccacaggtgtgcactccgacgtgcagcttcaggagtcaggacctggcctcgtgaaaccttctcagagtctgtccctcacctgt
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gaggatgacccagatgtccagatcagctggtttgtgaacaacgtggaagtacacacagctcagacacaaacccatagagaggattacaacagtactctccgggtggtcagtgccctccccatc
cagcaccaggactggatgagtggcaaggagttcaaatgcaaggtcaacaacaaagacctcccagcgcccatcgagagaaccatctcaaaacccaaagggtcagtaagagctccacaggtatat
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tacaagaacactgaaccagtcctggactctgatggttcttacttcatgtacagcaagctgagagtggaaaagaagaactgggtggaaagaaatagctactcctgttcagtggtccacgagggt
ctgcacaatcaccacacgactaagagcttctcccggactccgggtaaa

RX1 light chain nucleotide sequence:

atggtatccacacctcagttccttgtatttttgcttttctggattccagcctccagaggtgacatcttgctgactcagtctccagccatcctgtctgtgagtccaggagaaagagtcagtttc
tcctgcagggccagtcagagcattggcacaagcatacactggtatcagcaaagaacaaatggttctccaaggcttctcataaagtatgcttctgagtctatctctgggatcccttccaggttt
agtggcagtggatcagggacagattttactcttagcatcaacagtgtggagtctgaagatattgcagattattactgtcaacaaattaatagctggccaaccacgttcggcgggggacaaag
ttggaaataaaacgggctgatgctgcaccaactgtatccatcttcccaccatccagtgagcagttaacatctggaggtgcctcagtcgtgtgcttcttgaacaacttctaccccaaagacatc
aatgtcaagtggaagattgatggcagtgaacgacaaaatggcgtcctgaacagttggactgatcaggacagcaaagacagcacctacagcatgagcagcaccctcacgttgaccaaggacgag
tatgaacgacataacagctatacctgtgaggccactcacaagacatcaacttcacccattgtcaagagcttcaacaggaatgagtgt

FIG. 4B

CHIR-R1X Light Chain Risk Assignments

V-Region	Amino Acids 1-52
Risk	LHLHLHLMLLMLHLMLLLHLHLHLHMHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHH
Mouse	DILLTQSPAILSVSPGERVSFSCRASQSIGTSIH----WYQQRTNGSPRLLIKYAS

V-Region	Amino Acids 53-109
Risk	HLMMLHMLMHLHLHLHLLHLHLHLLHLLLHLHHHHHHHHHHHHHHHHHHHHHHLHHLHLLLLLL
Mouse	ESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQINSWPT------TFGGGTKLEI-KRA

FIG. 4C

CHIR-RX1Heavy Chain Risk Assignments

V-Region	Amino Acids 1-57
Risk	MHLHLHLHMLLMLMLLLHLHLHLHMHHHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHHHHHHHHHH
Mouse	DVQLQESGPGLVKPSQSLSLTCTVTDYSITSDYAWN-WIRQFPGNKLEWMGYIS---YSGST

V-Region	Amino Acids 58-113
Risk	HMHMMHMLMHLHLHLMLLMLHLHLHLLLHLLLLHLHMHHHHHHHHHHHHHHHHHHHHHHHHHHHHLHHLHLHLL
Mouse	SYNPSLKSRISITRDTSKNQFFLQLNSVTTEDTATYYCASFDYAHAM----------DYWGQGTSVTVSS

Atty. Docket No. 27527/40123
Inventors: Zimmerman et al.
Title: M-CSF-Specific Monoclonal Antibody and Uses Thereof
Sheet 6 of 33

Fig. 5

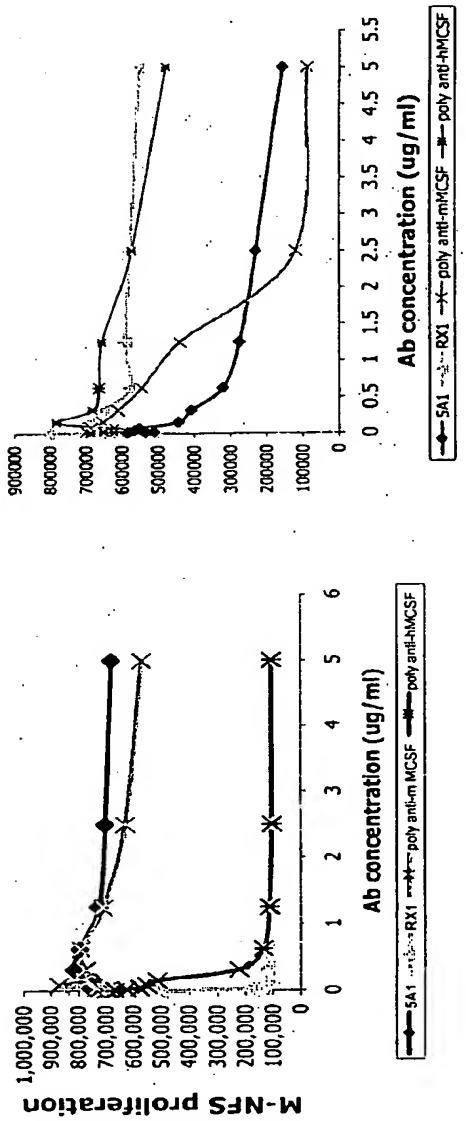

M-NFS proliferation
1,000,000
900,000
800,000
700,000
600,000
500,000
400,000
300,000
200,000
100,000
0
0 1 2 3 4 5 6
Ab concentration (ug/ml)
5A1
RX1
poly anti-m MCSF
poly anti-hMCSF
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
Ab concentration (ug/ml)
5A1
RX1
poly anti-mMCSF
poly anti-hMCSF

Fig. 6

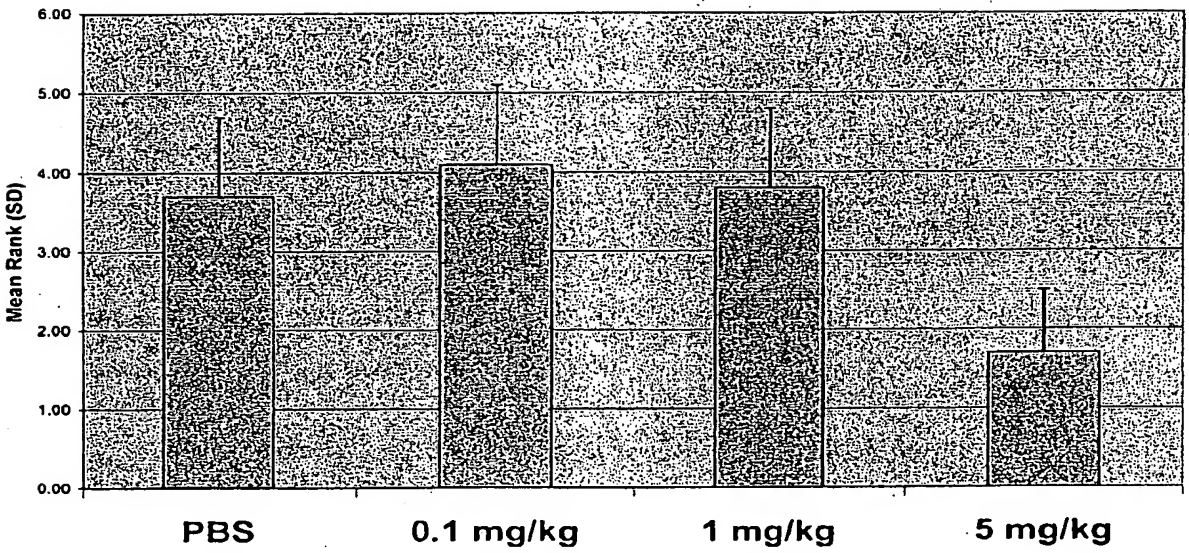

6.00
5.00
4.00
3.00
2.00
1.00
0.00
Mean Rank (SD)
PBS
0.1 mg/kg
1 mg/kg
5 mg/kg

Fig. 7

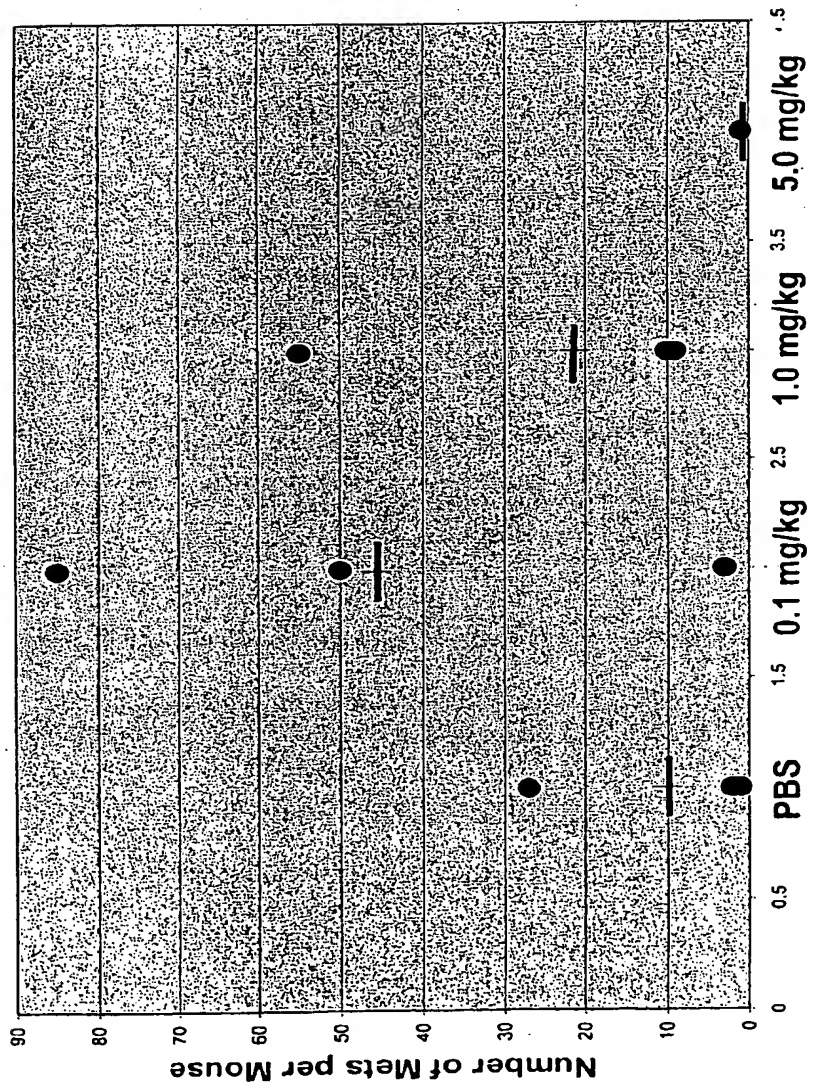

Number of Mets per Mouse
0
10
20
30
40
50
60
70
80
90
PBS
0.1 mg/kg
1.0 mg/kg
5.0 mg/kg
0
0.5
1.5
2.5
3.5
4.5

FIG. 8A

Binding of MCSF-specific antibody to breast cancer cell line MDA231

Red: no antibody control
Black: M-CSF antibody 1 ug/ml
Green: M-CSF antibody 10 ug/ml
Blue: M-CSF antibody 50 ug/ml

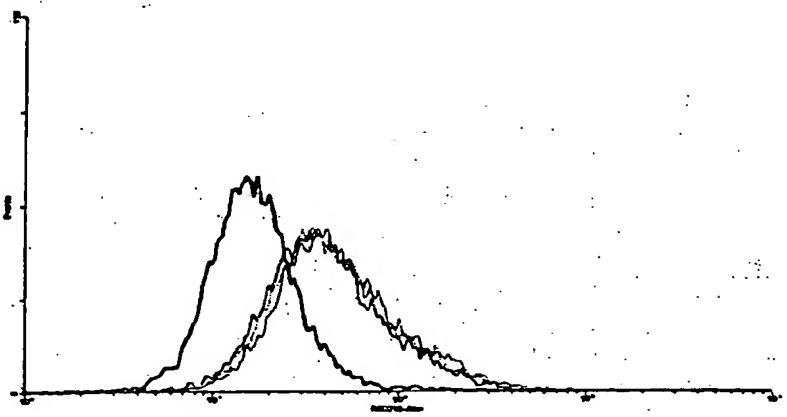


FIG. 8B

Binding of MCSF-specific antibody to multiple myeloma cancer cell line ARH77

Red: no antibody control
Green: M-CSF antibody 5 ug/ml
Blue: control IgG2a 5 ug/ml

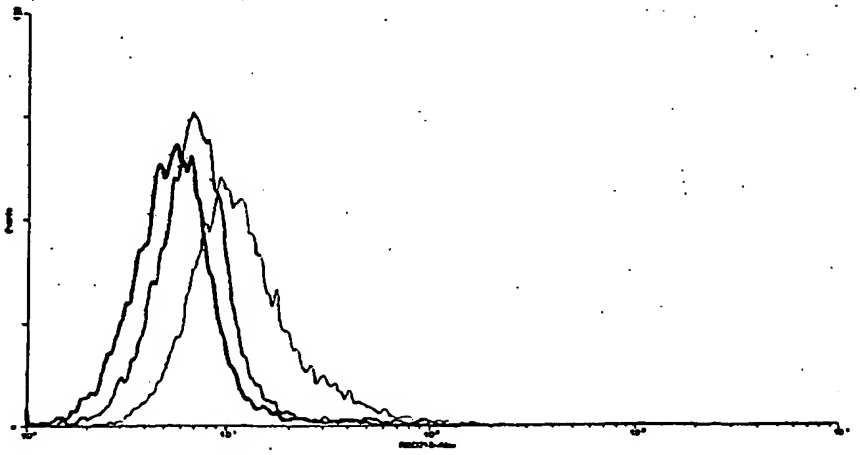


FIG. 9

Cancer Type	Cancer Status	Score 0	Score 1	Score 2	Score 3	Score 4	% with scores 3 or higher
adrenal	normal	10	5	5	0	0	0
basal cell	cancer	5	0	0	0	0	0
bladder	normal	6	1	2	1	0	10
brain	normal	17	1	2	0	0	0
breast	cancer	6	5	13	62	0	72
breast	normal	7	5	7	6	0	24
carcinoids	cancer	9	2	2	0	0	0
carcinoids (muscle)	cancer	1	0	1	0	0	0
choriocarcinoma	cancer	1	0	0	0	0	0
colon	normal	4	0	2	0	0	0
colon	cancer	9	0	1	4	0	27
fibrosarcoma	cancer	3	1	0	0	0	0
gallbladder	normal	2	1	0	1	0	25
germ cell	cancer	1	0	0	0	0	0
heart	normal	7	3	2	4	0	25
kidney	normal	5	10	1	4	0	20
kidney	cancer	8	1	0	3	0	25
leiomyosarcoma	cancer	5	0	0	0	0	0
liver	normal	11	3	4	1	0	5
liver	cancer	5	3	0	3	0	27
lung	normal	19	0	1	0	0	0
lung	cancer	3	1	0	3	0	43
lymphoma	cancer	13	0	3	2	0	12
melanoma	cancer	7	0	2	5	0	36
melanoma(inflammation)	cancer	0	0	0	1	0	100
mesothelioma	cancer	6	0	0	0	0	0
neuroblastoma	cancer	1	0	0	0	0	0
ovary	normal	6	0	2	0	0	0
ovary	cancer	8	2	0	4	0	29
pancreas	normal	9	2	5	4	0	20
pancreas	cancer	8	1	0	3	0	25
prostate	normal	0	3	8	3	0	21
prostate	cancer	9	1	1	4	0	27
sarcoma all	cancer	6	0	2	2	0	20
sarcoma	cancer	3	0	2	1	0	17
sarcoma (kidney)	cancer	3	0	2	1	0	17
sarcoma mfh	cancer	2	0	0	0	0	0
seminoma	cancer	3	0	0	0	0	0
small intestine	normal	2	1	0	1	0	25
spleen	normal	14	2	3	0	0	0
squamous cell	cancer	3	0	0	0	0	0
stomach	normal	3	2	2	1	0	13
stomach	cancer	7	1	1	1	0	10
teratoma	cancer	1	0	0	0	0	0
testis	normal	5	1	3	3	0	25
thyroid	normal	15	0	0	0	0	0
thyroid	cancer	6	2	1	2	0	18
undiff all	cancer	6	0	2	1	0	11
undif	cancer	5	0	2	0	0	0

Fig. 10

```
Met Thr Ala Pro Gly Ala Ala Gly Arg Cys Pro Pro Thr Thr Trp Leu
1               5                  10                  15

Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr
            20                  25                  30

Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu
        35                  40                  45

Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln
    50                  55                  60

Ile Thr Phe Glu Phe Val Asp Gln Glu Gln Leu Lys Asp Pro Val Cys
65                  70                  75                  80

Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu Asp Thr
                85                  90                  95

Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu
            100                 105                 110

Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu
        115                 120                 125

Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln
    130                 135                 140

Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu
145                 150                 155                 160

Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala
                165                 170                 175

Glu Cys Ser Ser Gln Gly His Glu Arg Gln Ser Glu Gly Ser Ser Ser
            180                 185                 190

Pro Gln Leu Gln Glu Ser Val Phe His Leu Leu Val Pro Ser Val Ile
        195                 200                 205

Leu Val Leu Leu Ala Val Gly Gly Leu Leu Phe Tyr Arg Trp Arg Arg
    210                 215                 220

Arg Ser His Gln Glu Pro Gln Arg Ala Asp Ser Pro Leu Glu Gln Pro
225                 230                 235                 240

Glu Gly Ser Pro Leu Thr Gln Asp Asp Arg Gln Val Glu Leu Pro Val
                245                 250                 255
```

Fig. 11

```
Met Thr Ala Pro Gly Ala Ala Gly Arg Cys Pro Pro Thr Thr Trp Leu
1               5                  10                  15
Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr
            20                  25                  30
Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu
        35                  40                  45
Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln
    50                  55                  60
Ile Thr Phe Glu Phe Val Asp Gln Glu Gln Leu Lys Asp Pro Val Cys
65                  70                  75                  80
Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu Asp Thr
                85                  90                  95
Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu
            100                 105                 110
Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu
        115                 120                 125
Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln
    130                 135                 140
Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu
145                 150                 155                 160
Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala
                165                 170                 175
Glu Cys Ser Ser Gln Asp Val Val Thr Lys Pro Asp Cys Asn Cys Leu
            180                 185                 190
Tyr Pro Lys Ala Ile Pro Ser Ser Asp Pro Ala Ser Val Ser Pro His
        195                 200                 205
Gln Pro Leu Ala Pro Ser Met Ala Pro Val Ala Gly Leu Thr Trp Glu
    210                 215                 220
Asp Ser Glu Gly Thr Glu Gly Ser Ser Leu Leu Pro Gly Glu Gln Pro
225                 230                 235                 240
Leu His Thr Val Asp Pro Gly Ser Ala Lys Gln Arg Pro Pro Arg Ser
                245                 250                 255
Thr Cys Gln Ser Phe Glu Pro Pro Glu Thr Pro Val Val Lys Asp Ser
            260                 265                 270
Thr Ile Gly Gly Ser Pro Gln Pro Arg Pro Ser Val Gly Ala Phe Asn
        275                 280                 285
Pro Gly Met Glu Asp Ile Leu Asp Ser Ala Met Gly Thr Asn Trp Val
    290                 295                 300
Pro Glu Glu Ala Ser Gly Glu Ala Ser Glu Ile Pro Val Pro Gln Gly
305                 310                 315                 320
Thr Glu Leu Ser Pro Ser Arg Pro Gly Gly Gly Ser Met Gln Thr Glu
                325                 330                 335
Pro Ala Arg Pro Ser Asn Phe Leu Ser Ala Ser Ser Pro Leu Pro Ala
            340                 345                 350
Ser Ala Lys Gly Gln Gln Pro Ala Asp Val Thr Gly Thr Ala Leu Pro
        355                 360                 365
Arg Val Gly Pro Val Arg Pro Thr Gly Gln Asp Trp Asn His Thr Pro
    370                 375                 380
Gln Lys Thr Asp His Pro Ser Ala Leu Leu Arg Asp Pro Pro Glu Pro
385                 390                 395                 400
Gly Ser Pro Arg Ile Ser Ser Leu Arg Pro Gln Gly Leu Ser Asn Pro
                405                 410                 415
Ser Thr Leu Ser Ala Gln Pro Gln Leu Ser Arg Ser His Ser Ser Gly
            420                 425                 430
Ser Val Leu Pro Leu Gly Glu Leu Glu Gly Arg Arg Ser Thr Arg Asp
        435                 440                 445
Arg Arg Ser Pro Ala Glu Pro Glu Gly Gly Pro Ala Ser Glu Gly Ala
    450                 455                 460
Ala Arg Pro Leu Pro Arg Phe Asn Ser Val Pro Leu Thr Asp Thr Gly
465                 470                 475                 480
His Glu Arg Gln Ser Glu Gly Ser Ser Ser Pro Gln Leu Gln Glu Ser
                485                 490                 495
Val Phe His Leu Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val
            500                 505                 510
Gly Gly Leu Leu Phe Tyr Arg Trp Arg Arg Arg Ser His Gln Glu Pro
        515                 520                 525
Gln Arg Ala Asp Ser Pro Leu Glu Gln Pro Glu Gly Ser Pro Leu Thr
    530                 535                 540
Gln Asp Asp Arg Gln Val Glu Leu Pro Val
```

Fig. 12

```
Met Thr Ala Pro Gly Ala Ala Gly Arg Cys Pro Pro Thr Thr Trp Leu
1               5                  10                  15
Gly Ser Leu Leu Leu Leu Val Cys Leu Leu Ala Ser Arg Ser Ile Thr
            20                  25                  30
Glu Glu Val Ser Glu Tyr Cys Ser His Met Ile Gly Ser Gly His Leu
        35                  40                  45
Gln Ser Leu Gln Arg Leu Ile Asp Ser Gln Met Glu Thr Ser Cys Gln
    50                  55                  60
Ile Thr Phe Glu Phe Val Asp Gln Glu Gln Leu Lys Asp Pro Val Cys
65                  70                  75                  80
Tyr Leu Lys Lys Ala Phe Leu Leu Val Gln Asp Ile Met Glu Asp Thr
                85                  90                  95
Met Arg Phe Arg Asp Asn Thr Pro Asn Ala Ile Ala Ile Val Gln Leu
            100                 105                 110
Gln Glu Leu Ser Leu Arg Leu Lys Ser Cys Phe Thr Lys Asp Tyr Glu
        115                 120                 125
Glu His Asp Lys Ala Cys Val Arg Thr Phe Tyr Glu Thr Pro Leu Gln
    130                 135                 140
Leu Leu Glu Lys Val Lys Asn Val Phe Asn Glu Thr Lys Asn Leu Leu
145                 150                 155                 160
Asp Lys Asp Trp Asn Ile Phe Ser Lys Asn Cys Asn Asn Ser Phe Ala
                165                 170                 175
Glu Cys Ser Ser Gln Asp Val Val Thr Lys Pro Asp Cys Asn Cys Leu
            180                 185                 190
Tyr Pro Lys Ala Ile Pro Ser Ser Asp Pro Ala Ser Val Ser Pro His
        195                 200                 205
Gln Pro Leu Ala Pro Ser Met Ala Pro Val Ala Gly Leu Thr Trp Glu
    210                 215                 220
Asp Ser Glu Gly Thr Glu Gly Ser Ser Leu Leu Pro Gly Glu Gln Pro
225                 230                 235                 240
Leu His Thr Val Asp Pro Gly Ser Ala Lys Gln Arg Pro Pro Arg Ser
                245                 250                 255
Thr Cys Gln Ser Phe Glu Pro Pro Glu Thr Pro Val Val Lys Asp Ser
            260                 265                 270
Thr Ile Gly Gly Ser Pro Gln Pro Arg Pro Ser Val Gly Ala Phe Asn
        275                 280                 285
Pro Gly Met Glu Asp Ile Leu Asp Ser Ala Met Gly Thr Asn Trp Val
    290                 295                 300
Pro Glu Glu Ala Ser Gly Glu Ala Ser Glu Ile Pro Val Pro Gln Gly
305                 310                 315                 320
Thr Glu Leu Ser Pro Ser Arg Pro Gly Gly Gly Ser Met Gln Thr Glu
                325                 330                 335
Pro Ala Arg Pro Ser Asn Phe Leu Ser Ala Ser Ser Pro Leu Pro Ala
            340                 345                 350
Ser Ala Lys Gly Gln Gln Pro Ala Asp Val Thr Gly His Glu Arg Gln
        355                 360                 365
Ser Glu Gly Ser Ser Ser Pro Gln Leu Gln Glu Ser Val Phe His Leu
    370                 375                 380
Leu Val Pro Ser Val Ile Leu Val Leu Leu Ala Val Gly Gly Leu Leu
385                 390                 395                 400
Phe Tyr Arg Trp Arg Arg Arg Ser His Gln Glu Pro Gln Arg Ala Asp
                405                 410                 415
Ser Pro Leu Glu Gln Pro Glu Gly Ser Pro Leu Thr Gln Asp Asp Arg
            420                 425                 430
Gln Val Glu Leu Pro Val
```

FIG. 13

5H4 heavy chain protein sequence:

```
  1   EIQLQQSGPE LVKTGTSVKI SCKASGYSFT GYFMHWVKQS HGKSLEWIGY
 51   ISCYNGDTNY NQNFKGKATF TVDTSSSTAY MQFNSLTSED SAVYYCAREG
101   GNYPAYWGQG TLVTVSAAKT TPPSVYPLAP GSAAQTNSMV TLGCLVKGYF
151   PEPVTVTWNS GSLSSGVHTF PAVLQSDLYT LSSSVTVPSS TWPSETVTCN
201   VAHPASSTKV DKKIVPRDCG CKPCICTVPE VSSVFIFPPK PKDVLTITLT
251   PKVTCVVVDI SKDDPEVQFS WFVDDVEVHT AQTQPREEQF NSTFRSVSEL
301   PIMHQDWLNG KEFKCRVNSA AFPAPIEKTI SKTKGRPKAP QVYTIPPPKE
351   QMAKDKVSLT CMITDFFPED ITVEWQWNGQ PAENYKNTQP IMDTDGSYFV
401   YSKLNVQKSN WEAGNTFTCS VLHEGLHNHH TEKSLSHSPG K
```

5H4 light chain protein sequence:

```
  1   DIVMTQSHKF MSTSVGDRVT ITCKASQNVG TAVTWYQQKP GQSPKLLIYW
 51   TSTRHAGVPD RFTGSGSGTD FTLTISDVQS EDLADYFCQQ YSSYPLTFGA
101   GTKLELKRAD AAPTVSIFPP SSEQLTSGGA SVVCFLNNFY PKDINVKWKI
151   DGSERQNGVL NSWTDQDSKD STYSMSSTLT LTKDEYERHN SYTCEATHKT
201   STSPIVKSFN RNEC
```

FIG. 14

MC-1 heavy chain protein sequence:

```
  1   EVKLVESGGG LVQPGGSLKL SCATSGFTFS DYYMYWVRQT PEKRLEWVAY
 51   ISNGGGSTYY PDTVKGRFTI SRDNAKNTLY LQMSRLKSED TAMYYCARQG
101   SYGYPFAYWG QGTLVTVSAA KTTAPSVYPL APVCGDTTGS SVTLGCLVKG
151   YFPEPVTLTW NSGSLSSGVH TFPAVLQSDL YTLSSSVTVT SSTWPSQSIT
201   CNVAHPASST KVDKKIEPRG PTIKPCPPCK CPAPNLLGGP SVFIFPPKIK
251   DVLMISLSPI VTCVVVDVSE DDPDVQISWF VNNVEVHTAQ TQTHREDYNS
301   TLRVVSALPI QHQDWMSGKE FKCKVNNKDL PAPIERTISK PKGSVRAPQV
351   YVLPPPEEEM TKKQVTLTCM VTDFMPEDIY VEWTNNGKTE LNYKNTEPVL
401   DSDGSYFMYS KLRVEKKNWV ERNSYSCSVV HEGLHNHHTT KSFSRTPGK
```

MC-1 light chain protein sequence:

```
  1   AIQMTQTTSS LSASLGDRVT ISCSASQGIS NYLNWYQQKP DGTVKLLIYY
 51   TSSLHSGVPS RFSGSGSGTD YSLTISNLEP EDIATYYCQQ YSKLPWTFGG
101   GTKLEIKRAD AAPTVSIFPP SSEQLTSGGA SVVCFLNNFY PKDINVKWKI
151   DGSERQNGVL NSWTDQDSKD STYSMSSTLT LTKDEYERHN SYTCEATHKT
201   STSPIVKSFN RNEC
```

FIG. 15

MC-3 heavy chain protein sequence:

```
  1   DVQLQESGPG LVKPSQSLSL TCTVTGYSIT SDYAWNWIRQ FPGNKLEWMG
 51   YISYSGSTSY NPSLKSRISI TRDTSKNQFF LQLNSVTTED TATYYCARLE
101   TWLFDYWGQG TTLTVSSAKT TPPSVYPLAP GCGDTTGSSV TLGCLVKGYF
151   PESVTVTWNS GSLSSSVHTF PALLQSGLYT MSSSVTVPSS TWPSQTVTCS
201   VAHPASSTTV DKKLEPSGPI STINPCPPCK ECHKCPAPNL EGGPSVFIFP
251   PNIKDVLMIS LTPKVTCVVV DVSEDDPDVQ ISWFVNNVEV HTAQTQTHRE
301   DYNSTIRVVS TLPIQHQDWM SGKEFKCKVN NKDLPSPIER TISKIKGLVR
351   APQVYILPPP AEQLSRKDVS LTCLVVGFNP GDISVEWTSN GHTEENYKDT
401   APVLDSDGSY FIYSKLNMKT SKWEKTDSFS CNVRHEGLKN YYLKKTISRS
451   PGLDLDDICA EAKDGELDGL WTTITIFISL FLLSVCYSAS VTLFKVKWIF
501   SSVVELKQKI SPDYRNMIGQ GA
```

MC-3 light chain protein sequence:

```
  1   DILLTQSPAI LSVSPGERVS FSCRASQSIG TSIHWYQQRT NGSPRLLIKY
 51   ASESISGIPS RFSGSGSGTD FTLSINSVES EDIADYYCQQ SNSWPTTFGG
101   GTKLEIKWAD AAPTVSIFPP SSEQLTSGGA SVVCFLNNFY PKDINVKWKI
151   DGSERQNGVL NSWTDQDSKD STYSMSSTLT LTKDEYERHN SYTCEATHKT
201   STSPIVKSFN RNEC
```

FIG. 16A

For heavy chain CDR1:

```
                    1
     H_CDR1_5H4  (1) -GYFMH
     H_CDR1_MC-1 (1) -DYYMY
H_CDR1_CHIR-RX1  (1) SDYAWN
     H_CDR1_MC-3 (1) SDYAWN
       Consensus (1) SDYAWN
```

For heavy chain CDR2:

```
                    1                 17
     H_CDR2_5H4  (1) YISCYNGDTNYNQNFKG
     H_CDR2_MC-1 (1) YISNGGGSTYYPDTVKG
H_CDR2_CHIR-RX1  (1) YIS-YSGSTSYNPSLKS
     H_CDR2_MC-3 (1) YIS-YSGSTSYNPSLKS
       Consensus (1) YIS YSGSTSYNPSLKS
```

For heavy chain CDR3:

```
                    1
     H_CDR3_5H4  (1) --EGGNYPAY
     H_CDR3_MC-1 (1) QGSYGYPEAY
H_CDR3_CHIR-RX1  (1) -FDYAHAMDY
     H_CDR3_MC-3 (1) --LETWLEWY
       Consensus (1)   DYGW FDY
```

FIG. 16B

For light chain CDR1:

```
                          1          11
      L_CDR1_5H4    (1)  KASQNVGTAVT
     L_CDR1_MC-1    (1)  SASQGISNYLN
L_CDR1_CHIR-RX1     (1)  RASQSIGTSIH
     L_CDR1_MC-3    (1)  RASQSIGTSIH
       Consensus    (1)  RASQSIGTSIH
```

For light chain CDR2:

```
                          1
      L_CDR2_5H4    (1)  WTSTRHA
     L_CDR2_MC-1    (1)  YTSSLHS
L_CDR2_CHIR-RX1     (1)  YASESIS
     L_CDR2_MC-3    (1)  YASESIS
       Consensus    (1)  YTSESIS
```

For light chain CDR3:

```
                          1
      L_CDR3_5H4    (1)  QQYSSYPLT
     L_CDR3_MC-1    (1)  QQYSKLPWT
L_CDR3_CHIR-RX1     (1)  QQINSWPTT
     L_CDR3_MC-3    (1)  QQSNSWPTT
       Consensus    (1)  QQYSSWPTT
```

FIG. 17

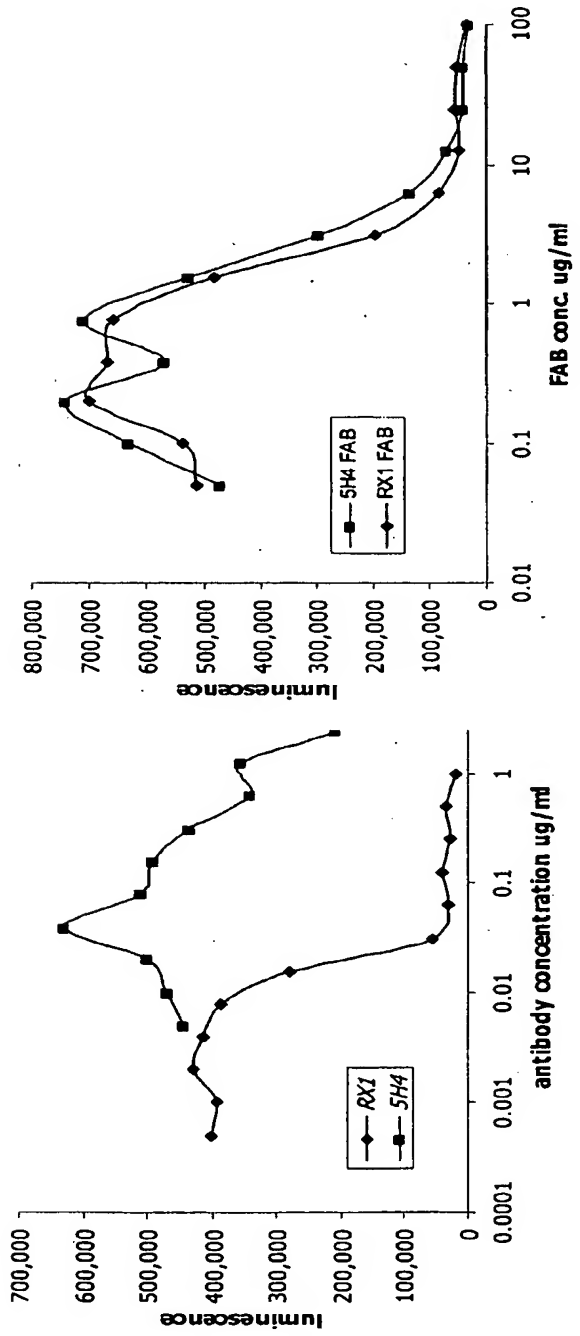

Neutralization Activities of Intact mAbs vs. Fabs
Intact mAb
Fab
luminescence
antibody concentration ug/ml
FAB conc. ug/ml
RX1
5H4
5H4 FAB
RX1 FAB

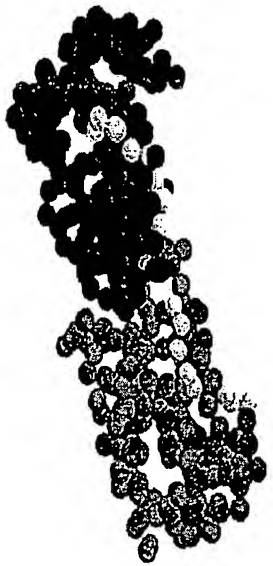
FIG. 18

MCSF Structure with RX1 Epitopes Highlighted

RX1

$R_{98}FRDNTPN_{105}$

5H4



$I_{65}TFEFVDQE_{73}$

MC3

$I_{65}TFEFVDQE_{73}$

$F_{138}YETPLQ_{144}$

FIG. 19A

Heavy Chain

V-Region	No.of Changes	Amino Acids 1-57
Risk		MHLHLHLHMLLMLMLLLHLHLHLHMHHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHHHHHHHH
Mouse		DVQLQESGPGLVKPSQSLSLTCTVTDYSITSDYAWN-WIRQFPGNKLEWMGYIS---YSGST
Human		qvqLqesGpgLVkPsqTLsLTCxvsGxsxSsxxxxxxWiRQpPgkgLEWigxiyyraxxgxt
Low Risk	2	DVQLQESGPGLVKPSQTLSLTCTVTDYSITSDYAWN-WIRQFPGKKLEWMGYIS---YSGST
Low+Mod	5	QVQLQESGPGLVKPSQTLSLTCTVSDYSITSDYAWN-WIRQFPGKGLEWMGYIS---YSGST

V-Region	No.of Changes	Amino Acids 58-113
Risk		HMHMMHMLMHLHLHLMLLMLHLHLHLLHLLLLHLHMHHHHHHHHHHHHHHHHHHHHHHHHHHHLHHLHLHLL
Mouse		SYNPSLKSRISITRDTSKNQFFLQLNSVTTEDTATYYCASFDYAHAM----------DYWGQGTSVTVSS
Human		xynpSlksRvTisvDTSKNQfsLxlxsvtaaDTAvYyCArxxxxxxxxxxxxxxxxxfdxWGqGtxVTVSS
Low Risk	6	SYNPSLKSRITISRDTSKNQFSLQLNSVTAADTATYYCASFDYAHAM----------DYWGQGTTVTVSS
Low+Mod	7	SYNPSLKSRITISRDTSKNQFSLQLNSVTAADTAVYYCASFDYAHAM----------DYWGQGTTVTVSS

FIG. 19B

Low Risk Heavy Chain Vs. Kabat Vh2 Consensus:

Protein Seq:

DVQLQESGPGLVKPSQTLSLTCTVTDYSITSDYAWNWIRQFPGKKLEWMGYISYSGSTSYNPSLKSRITISRDTSKNQFSLQLNSVTAADTATYYCASFDYAHAMD
YWGQGTTVTVSS

DNA Seq:

GACGTACAACTTCAAGAATCTGGCCCAGGTCTCGTCAAACCTTCTCAAACTCTCTCACTCACCTGCACTGTTACTGACTACTCTATTACATCCGACTACGCTT
GGAACTGGATCCGACAATTTCCTGGTAAAAAACTCGAATGGATGGGTTATATTTCTTACTCTGGCTCCACCTCCTACAATCCTTCTCTGAAATCACGCATCAC
AATTTCCCGCGATACCTCTAAAAATCAATTTTCACTCCAACTCAATTCTGTTACCGCCGCCGATACTGCCACCTACTACTGTGCCTCTTTTGACTACGCTCACG
CCATGGATTATTGGGGACAGGGTACTACCGTTACCGTAAGCTCA

Low Risk + Moderate Risk Heavy Chain Vs. Kabat Vh2 Consensus:

Protein Seq:

QVQLQESGPGLVKPSQTLSLTCTVSDYSITSDYAWNWIRQFPGKGLEWMGYISYSGSTSYNPSLKSRITISRDTSKNQFSLQLNSVTAADTAVYYCASFDYAHAMD
YWGQGTTVTVSS

DNA Seq:

CAAGTTCAACTTCAAGAATCAGGCCCCGGACTCGTTAAACCCTCTCAAACTCTCTCTCTTACTTGCACTGTATCCGATTACTCTATTACTTCAGACTACGCTTG
GAACTGGATCAGACAATTTCCCGGAAAAGGACTCGAATGGATGGGATATATCTCTTACTCTGGCTCAACCTCTTACAACCCCTCTCTCAAATCTCGAATAAC
AATCTCACGCGATACTTCTAAAAATCAATTCTCACTTCAACTTAACTCCGTTACTGCCGCCGACACTGCCGTTTACTACTGTGCTTCCTTCGATTACGCCCACG
CTATGGATTATTGGGGACAAGGAACTACCGTCACTGTCAGCTCA

FIG. 20A

Light Chain

V-Region	No.of Changes	Amino Acids 1-52
Risk		LHLHLHLMLLMLHLMLLLHLHLHLHMHHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHH
Mouse		DILLTQSPAILSVSPGERVSFSCRASQSIGTSIH----WYQQRTNGSPRLLIKYAS
Human		EIVlTQSPgTLSlSPGERaTLSCRASQSvsssyL---AWYQQkPGQAPRLLIYgAS
Low Risk	8	EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIH----WYQQKTGQSPRLLIKYAS
Low+Mod	9	EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIH----WYQQKTGQAPRLLIKYAS

V-Region	No.of Changes	Amino Acids 53-109
Risk		HLMMLHMLMHLHLHLHLLLHLHLHLLHLLLLHLHHHHHHHHHHHHHHHHHHHHHHLHHLHLLLLLL
Mouse		ESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQINSWPT------TFGGGTKLEI-KRA
Human		sRATGIPdRFSGSGSGTDFTLTISrLepEDFAVYYCQQygsspp-----xTFGqGTKvEI-KRT
Low Risk	8	ERISGIPDRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPT------TFGQGTKLEI-KRT
Low+Mod	10	ERATGIPDRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPT------TFGQGTKLEI-KRT

FIG. 20B

Low Risk Light Chain Vs. Kabat Vk3 Consensus:

Protein Seq:

EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIHWYQQKTGQSPRLLIKYASERISGIPDRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPTTFGQGTKLEIKRT

Nucleotide Seq:

GAAATAGTCCTTACCCAATCTCCCGGAACCCTCTCAGTATCTCCCGGCGAACGAGTAACCTTTTCATGTAGAGCATCCCAATCCATCGGCACTTCAATTCACT
GGTATCAGCAGAAAACAGGTCAATCCCCACGGCTTCTTATAAAATATGCATCAGAAAGAATATCAGGCATTCCAGACAGATTCTCAGGTTCAGGTTCAGGC
ACAGACTTCACACTTACAATTTCCCGCGTCGAATCCGAAGACTTCGCTGACTATTACTGCCAACAAATCAACTCATGGCCTACTACTTTCGGTCAAGGCACC
AAACTCGAAATTAAACGTACG

Low Risk + Moderate Risk Light Chain Vs. Kabat Vk3 Consensus:

Protein Seq:

EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIHWYQQKTGQAPRLLIKYASERATGIPDRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPTTFGQGTKLEIKRT

Nucleotide Seq:

GAAATAGTTCTTACTCAATCCCCCGGTACACTCTCAGTTTCCCCAGGCGAACGCGTCACTTTTTCTTGCAGAGCATCACAATCAATCGGCACTTCAATTCATT
GGTATCAACAAAAAACAGGACAGGCCCCACGACTTCTTATTAAATATGCATCAGAACGAGCCACAGGCATCCCAGACAGATTTTCAGGTTCAGGATCAGGC
ACCGATTTCACACTTACAATATCCAGAGTCGAATCAGAAGATTTTGCAGATTACTATTGTCAACAAATAAACAGCTGGCCCACTACATTCGGACAAGGCACA
AAACTCGAAATTAAACGTACG

FIG. 21A

Light Chain – Changes back to Murine

V-Region	No.of Changes	Amino Acids 1-52
Risk		LHLHLHLMLLMLHLMLLLHLHLHLHMHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHH
Mouse		DILLTQSPAILSVSPGERVSFSCRASQSIGTSIH----WYQQRTNGSPRLLIKYAS
Human		EIVlTQSPgTLSlSPGERaTLSCRASQSvsssyL---AWYQQkPGQAPRLLIYgAS
Low Risk	8	EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIH----WYQQKTGQSPRLLIKYAS
Low+Mod	9	EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIH----WYQQKTGQAPRLLIKYAS

V-Region	No.of Changes	Amino Acids 53-109
Risk		HLMMLHMLMHLHLHLHLLHLHLHLLHLLLHLHHHHHHHHHHHHHHHHHHHHHHLHHLHLLLLLL
Mouse		ESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQINSWPT------TFGGGTKLEI-KRA
Human		sRATGIPdRFSGSGSGTDFTLTISrLepEDFAVYYCQQygsspp-----xTFGqGTKvEI-KRT
Low Risk	8	ERISGIPDRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPT------TFGQGTKLEI-KRT
Low+Mod	10	ERATGIPDRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPT------TFGQGTKLEI-KRT
Low+Mod Altnernate	7	ESISGIPDRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPT------TFGQGTKLEI-KRT

FIG. 21B

Low Risk Light Chain Vs. Kabat Vk3 Consensus; AA54 changed back to murine:

Protein Seq:

EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIHWYQQKTGQSPRLLIKYASESISGIPDRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPTTFGQGTKLEIKRT

Nucleotide Seq:

GAAATAGTCCTTACCCAATCTCCCGGAACCCTCTCAGTATCTCCCGGCGAACGAGTAACCTTTTCATGTAGAGCATCCCAATCCATCGGCACTTCAATTCACT
GGTATCAGCAGAAAACAGGTCAATCCCCACGGCTTCTTATAAAATATGCATCAGAATCAATTTCTGGCATCCCAGACAGATTTTCAGGTTCAGGATCAGGCA
CCGATTTCACACTTACAATATCCAGAGTCGAATCAGAAGATTTTGCAGATTACTATTGTCAACAAATAAACAGCTGGCCCACTACATTCGGACAAGGCACAA
AACTCGAAATTAAACGTACG

Low Risk + Moderate Risk Light Chain Vs. Kabat Vk3 Consensus; AA54, 55, 56 changed back to murine:

Protein Seq:

EIVLTQSPGTLSVSPGERVTFSCRASQSIGTSIHWYQQKTGQAPRLLIKYASESISGIPDRFSGSGSGTDFTLTISRVESEDFADYYCQQINSWPTTFGQGTKLEIKRT

Nucleotide Seq:

GAAATAGTTCTTACTCAATCCCCCGGTACACTCTCAGTTTCCCCAGGCGAACGCGTCACTTTTTCTTGCAGAGCATCACAATCAATCGGCACTTCAATTCATT
GGTATCAACAAAAAACAGGACAGGCCCCACGACTTCTTATTAAATATGCATCAGAATCAATTTCTGGCATCCCAGACAGATTTTCAGGTTCAGGATCAGGCA
CCGATTTCACACTTACAATATCCAGAGTCGAATCAGAAGATTTTGCAGATTACTATTGTCAACAAATAAACAGCTGGCCCACTACATTCGGACAAGGCACAA
AACTCGAAATTAAACGTACG

FIG. 22A

Light Chain – Changes based on HK6 2-1-1(A14)

V-Region	No.of Changes	Amino Acids 1-52
Risk		LHLHLHLMLLMLHLMLLLHLHLHLHMHHHHHHHHHHHHHHHMHLMLLMHMHHHHHHH
Mouse		DILLTQSPAILSVSPGERVSFSCRASQSIGTSIH----WYQQRTNGSPRLLIKYAS
Human		DVVMTQSPAFLSVTPGEKVTITCQASEGIGNYLY----WYQQKPDQAPKLLIKYAS
Low Risk	10	DIVLTQSPAFLSVTPGEKVTFTCQASQSIGTSIH----WYQQKTDQSPRLLIKYAS
Low+Mod	12	DIVLTQSPAFLSVTPGEKVTFTCQASQSIGTSIH----WYQQKTDQAPKLLIKYAS

V-Region	No.of Changes	Amino Acids 53-109
Risk		HLMMLHMLMHLHLHLHLLLHLHLHLLHLLLHLHHHHHHHHHHHHHHHHHHHHHLHHLHLLLLLL
Mouse		ESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQINSWPT------TFGGGTKLEI-KRA
Human		QSISGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQQGNKHP------LTFGGGTKVEI-KRT
Low Risk	5	ESISGIPSRFSGSGSGTDFTLTISSVEAEDAADYYCQQINSWPT------TFGGGTKLEI-KRT
Low+Mod	5	ESISGIPSRFSGSGSGTDFTLTISSVEAEDAADYYCQQINSWPT------TFGGGTKLEI-KRT

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Title: M-CSF-Specific Monoclonal Antibody and Uses Thereof
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FIG. 22B

Low Risk Light Chain vs. VK6 Subgroup 2-1-(1) A14:

Protein Seq:

DIVLTQSPAFLSVTPGEKVTFTCQASQSIGTSIHWYQQKTDQSPRLLIKYASESISGIPSRFSGSGSGTDFTLTISSVEAEDAADYYCQQINSWPTTFGGGTKLEIKRT

Nucleotide Seq: Not synthesized

Low Risk + Moderate Risk Light Chain vs. VK6 Subgroup 2-1-(1) A14:

DIVLTQSPAFLSVTPGEKVTFTCQASQSIGTSIHWYQQKTDQAPKLLIKYASESISGIPSRFSGSGSGTDFTLTISSVEAEDAADYYCQQI
NSWPTTFGGGTKLEIKRT

Nucleotide Seq:

GACATAGTTCTCACACAATCACCAGCATTCCTCTCAGTTACACCCGGCGAAAAAGTAACCTTTACCTGTCAGGCTTCTCAATCTATCGGCACTTCTATTCACT
GGTATCAACAAAAAACCGATCAAGCTCCTAAACTCCTCATAAAATACGCATCCGAATCCATCTCCGGTATCCCCTCCAGATTTTCAGGCTCCGGCTCCGGCA
CAGATTTCACCCTTACCATTAGCTCAGTTGAAGCCGAAGACGCAGCTGATTACTACTGTCAACAAATAAACTCATGGCCCACTACTTTCGGCGGCGGCACTA
AACTCGAAATAAAACGTACG

FIG. 23A

Murine RX-1 Light Chain:

DILLTQSPAILSVSPGERVSFSCRASQSIGTSIHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQINSWPTTFGGGTK
LEIKRA

```
        RX1 KV     (1)  DILLTQSPAILSVSPGERVSFSCRASQSIGT-S-----IHWYQQRTNGSPRLLIKYASESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQINSWPTTPG
Consensus Germline LC
hVK I Consensus    (1)  DIQMTQSPSSLSASVGDRVTITCRASQSISS-Y-----LNWYQQKPGKAPKLLIYAASSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTP----
hVK II Consensus   (1)  DIVMTQTPLSLPVTPGEPASISCRSSQSLLDSDDGNTYLDWYLQKPGQSPQLLIYTLSYRASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQRIEFP----
hVK III Consensus  (1)  EIVLTQSPGTLSLSPGERATLSCRASQSVSSSY-----LAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDPAVYYCQQYGSSP----
hVK IV Consensus   (1)  DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSTP----
hVK V Consensus    (1)  ETTLTQSPAFMSATPGDKVNISCKASQDIDD-D-----MNWYQQKPGEAAIFIQEATTLVPGIPPRFSGSGYGTDFTLTINNIESEDAAYYFCLQHDNFP----
hVK VI Consensus   (1)  EIVLTQSPDPQSVTPKEKVTITCRASQSIGS-S-----LHWYQQKPDQSPKLLIKYASQSPSGVPSRFSGSGSGTDFTLTINSLEAEDAATYYCHQSSSLP----
```

LIGHT CHAIN amino half

RX-1 DILLTQSPAILSVSPGERVSFSCRASQSI--GTSIH----WYQQRTNGSPRLLIKYAS

pos... 10 20 abcdef 30 40 50

Kabat:

HK1...DIQMTQSPSSLSASVGDRVTITCRASQSLVXX-XISXXLXWYQQKPGKAPKLLIYXAS
HK2...DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSXDGXXYLNWYLQKPGQSPQLLIYXXS
HK3...EIVLTQSPGTLSLSPGERATLSCRASQS-----VSSSYLAWYQQKPGQAPRLLIYGAS
HK4...DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIYWAS

Germline Consensus (with JK4):

hVK1 DIQMTQSPSSLSASVGDRVTITCRASQS------ISSYLNWYQQKPGKAPKLLIYAAS
hVK2 DIVMTQTPLSLPVTPGEPASISCRSSQSLLDSDDGNTYLDWYLQKPGQSPQLLIYTLS
hVK3 EIVLTQSPGTLSLSPGERATLSCRASQS-----VSSSYLAWYQQKPGQAPRLLIYGAS
hVK4 DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIYWAS
hVK5 ETTLTQSPAFMSATPGDKVNISCKASQDIDD------DMNWYQQKPGEAAIFIIQEAT
hVK6 EIVLTQSPDFQSVTPKEKVTITCRASQSIG------SSLHWYQQKPDQSPKLLIKYAS

FIG. 23B

LIGHT CHAIN carboxy half

```
RX-1  ESISGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQINSWPT------TFGGGTKLEI-KRA

pos...      60        70        80        90     abcdef  100       a
```

Kabat:

```
HK1...XLXSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQXXXXPE-----XTFGQGTKVEI-KRT
HK2...NRXSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQAXQXPR-----XTFGQGTKVEI-KRT
HK3...SRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPP-----XTFGQGTKVEI-KRT
HK4...TRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSTP------XTFGQGTKVEI-KRT
```

Germline Consensus (with JK4):

```
hVK1  SLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSYSTP------LTFGGGTKVEI-KRT
hVK2  YRASGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCMQRIEFP------LTFGGGTKVEI-KRT
hVK3  SRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSP------LTFGGGTKVEI-KRT
hVK4  TRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSTP------LTFGGGTKVEI-KRT
hVK5  TLVPGIPPRFSGSGYGTDFTLTINNIESEDAAYYFCLQHDNFP------LTFGGGTKVEI-KRT
hVK6  QSFSGVPSRFSGSGSGTDFTLTINSLEAEDAATYYCHQSSSLP------LTFGGGTKVEI-KRT
```

FIG. 24A

Murine RX-1 Heavy Chain:

DVQLQESGPGLVKPSQSLSLTCTVTDYSITSDYAWNWIRQFPGNKLEWMGYISYSGSTSYNPSLKSRISITRDTSKNQFFLQLNSVTTEDTATYYC
ASFDYAHAMDYWGQGTSVTVSS

```
RX1 VH              (1) D VQLQESGPGLVKPSQSLS LTCTVTDYSITS-DYAWNWIRQFPGNKLEWMGYISYSGSTS---YNPSLKSRISITRDTSKNQ FLQLNSVTTEDTATYYCASFDY
Consensus Germline
hVH I Consensus     (1)  QVQLVQSGAEVKKPGASVKVSCKASGYTFTG--YYMHWVRQAPGQGLEWMGWINP--NSGGTNYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCAR---
hVH II Consensus    (1)  QITLKESGPTLVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALEWLALIYWNDDKR---YSPSLKSRLTITKDTSKNQVVLTMTNMDPVDTATYYCAHR--
hVH III Consensus   (1)  E VQLVESGGGLVQPGGSLRLSCAASGFTFSS--YWMSWVRQAPGKGLEWVANIKQ--DGSEKYYVDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR---
hVH IV Consensus    (1)  QVQLQESGPGLVKPSGTLSLTCAVSGGSISSS-NWWSWVRQPPGKGLEWIGEIYHSGSTN---YNPSLKSRVTISVDKSKNQFSL KLSSVTAADTAVYYCAR---
hVH V Consensus     (1)  E VQLVQSGAEVKKPGESLKISCKGSGYSFTS--YWIGWVRQMPGKGLEWMGIIYP--GDSDTRYSPSFQGQVTISADKSIS TAYLQWSSLKASDTAMYYCAR---
hVH VI Consensus    (1)  QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYRSKWYN-DYAVSVKSRITINPDTSKNQFS LQLNSVTPEDTAVYYCAR---
hVH VII Consensus   (1)  QVQLVQSGSELKKPGASVKVSCKASGYTFTS--YAMNWVRQAPGQGLEWMGWINT--NTGNPTYAQGFTGRFVFSLDTSV STAYLQICSLKAEDTAVYYCAR---
```

HEAVY CHAIN amino half

```
      DVQLQESGPGLVKPSQSLSLTCTVTDYSITSDYAWN-WIRQFPGNKLEWMGYIS---YSGST

pos ...        10        20        30   ab   40        50  abc
```

Kabat:

```
HH1 ...XVQLVQSGAEVKKPGXSVKVSCKASGYTFXSYXIX--WVRQAPGQGLEWMGXIXPY-XXGXT
HH2 ...QVQLQESGPGLVKPSQTLSLTCXVSGXSXSSXXXXXXWIRQPPGKGLEWIGXIYYRAXXGXT
HH3 ...EVQLVESGGGLVQPGGSLRLSCAASGFTFSXYXMX--WVRQAPGKGLEWVXXIXXKXXGXXT
```

Germline Consensus (with JH4):

```
hVHI   QVQLVQSGAEVKKPGASVKVSCKASGYTFTGYYMH--WVRQAPGQGLEWMGWINP--NSGGT
hVHII  QITLKESGPTLVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALEWLALIY---WNDDK
hVHIII EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYWMS--WVRQAPGKGLEWVANIK--QDGSEK
hVHIV  QVQLQESGPGLVKPSGTLSLTCAVSGGSISSSNWW-SWVRQPPGKGLEWIGEIY---HSGST
hVHVV  EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIG--WVRQMPGKGLEWMGIIYP--GDSDT
hVHVI  QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYY-RSKWYN
hVHVII QVQLVQSGSELKKPGASVKVSCKASGYTFTSYAMN--WVRQAPGQGLEWMGWINT--NTGNP
```

FIG. 24B

HEAVY CHAIN carboxy half

```
      SYNPSLKSRISITRDTSKNQFFLQLNSVTTEDTATYYCASFDYAHAM----------DYWGQGTSVTVSS

pos ... 60        70        80  abc       90        100abcdefghijk        110

Kabat:

HH1 ...NYAQKFQGRVTITXDXSTSTAYMELSSLRSXDTAVYYCARXXXXXXXXXXXXXXDXXFDXWGQGTLVTVSS
HH2 ...XYNPSLKSRVTISVDTSKNQFSLXLXSVTAADTAVYYCARXXXXXXXXXXXXXXXXXFDXWGQGTXVTVSS
HH3 ...YYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAXXXXXXXXXXXXXXYYXXFDXWGQGTLVTVSS

Germline Consensus (with JH4):

hVHI   NYAQKFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCARXXXXXXXXXXXXXXXXYFDYWGQGTLVTVSS
hVHII  RYSPSLKSRLTITKDTSKNQVVLTMTNMDPVDTATYYCAHRXXXXXXXXXXXXXXXYFDYWGQGTLVTVSS
hVHIII YYVDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARXXXXXXXXXXXXXXXXYFDYWGQGTLVTVSS
hVHIV  NYNPSLKSRVTISVDKSKNQFSLKLSSVTAADTAVYYCARXXXXXXXXXXXXXXXXYFDYWGQGTLVTVSS
hVHV   RYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCARXXXXXXXXXXXXXXXXYFDYWGQGTLVTVSS
hVHVI  DYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCARXXXXXXXXXXXXXXXXYFDYWGQGTLVTVSS
hVHVII TYAQGFTGRFVFSLDTSVSTAYLQICSLKAEDTAVYYCARXXXXXXXXXXXXXXXXYFDYWGQGTLVTVSS
```

PATENT COOPERATION TREATY

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Date of mailing *(day/month/year)* 28 March 2005 (28.03.2005)	
Applicant's or agent's file reference 21601.003	**IMPORTANT NOTIFICATION**
International application No. PCT/US05/000546	International filing date *(day/month/year)* 06 January 2005 (06.01.2005)
International publication date *(day/month/year)*	Priority date *(day/month/year)* 07 January 2004 (07.01.2004)
Applicant CHIRON CORPORATION et al	

1. By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. *(If applicable)* The **letters "NR"** appearing in the right-hand column denote **a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau** under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

3. *(If applicable)* **An asterisk (*)** appearing next to a date of receipt, in the right-hand column, denotes **a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b)** (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
07 January 2004 (07.01.2004)	60/535,181	US	18 February 2005 (18.02.2005)
02 June 2004 (02.06.2004)	60/576,417	US	21 February 2005 (21.02.2005)

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